



## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

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<b>(54) Title:</b> ANTI-'alpha'v'beta'3 RECOMBINANT HUMAN ANTIBODIES, NUCLEIC ACIDS ENCODING SAME AND METHODS OF USE <b>(54) Titre:</b> ANTICORPS HUMAINS RECOMBINANTS ANTI-ALPHA <sub>v</sub> BETA <sub>3</sub> , ACIDES NUCLEIQUES CODANT CES ANTICORPS ET METHODES D'UTILISATION  <b>(57) Abstract</b> <p>The invention provides enhanced LM609 grafted antibodies exhibiting selective binding affinity to 'alpha'v'beta'3, or a functional fragment thereof. The invention also provides nucleic acid molecules encoding the enhanced LM609 grafted antibodies. Additionally provided are methods of inhibiting a function of 'alpha'v'beta'3 by contacting 'alpha'v'beta'3 with an enhanced LM609 grafted antibody.</p> <b>(57) Abrégé</b> <p>L'invention concerne des anticorps greffés LM609 améliorés ayant une affinité de liaison sélective pour 'alpha'v'beta'3, ou un fragment fonctionnel desdits anticorps. Elle concerne également des molécules d'acide nucléique codant lesdits anticorps greffés LM609 améliorés, ainsi que des méthodes qu'on met en oeuvre pour inhiber une fonction de 'alpha'v'beta'3 en plaçant 'alpha'v'beta'3 au contact d'un anticorps greffé LM609 amélioré.</p>		

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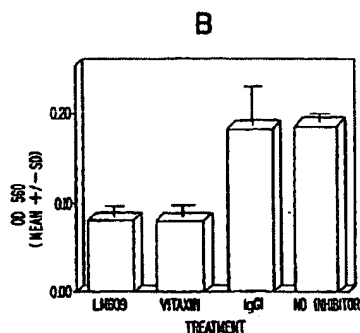
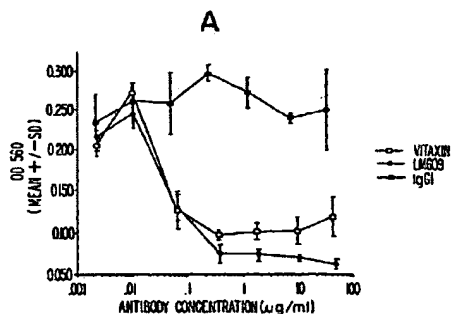
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[Continued on next page]

(54) Title: ANTI- $\alpha_3\beta_1$  RECOMBINANT HUMAN ANTIBODIES, NUCLEIC ACIDS ENCODING SAME AND METHODS OF USE



(57) Abstract: The invention provides enhanced LM609 grafted antibodies exhibiting selective binding affinity to  $\alpha_3\beta_1$ , or a functional fragment thereof. The invention also provides nucleic acid molecules encoding the enhanced LM609 grafted antibodies. Additionally provided are methods of inhibiting a function of  $\alpha_3\beta_1$  by contacting  $\alpha_3\beta_1$  with an enhanced LM609 grafted antibody.

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**Description**

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ANTI- $\alpha_v\beta_3$ , RECOMBINANT HUMAN ANTIBODIES, NUCLEIC ACIDS  
ENCODING SAME AND METHODS OF USE

BACKGROUND OF THE INVENTION

The present invention relates generally to integrin mediated diseases and, more particularly, to nucleic acids encoding  $\alpha_v\beta_3$ -inhibitory monoclonal antibodies and to CDR grafted  $\alpha_v\beta_3$ -inhibitory antibodies for the therapeutic treatment of  $\alpha_v\beta_3$ -mediated diseases.

Integrins are a class of cell adhesion receptors that mediate both cell-cell and cell-extracellular matrix adhesion events. Integrins consist of heterodimeric polypeptides where a single  $\alpha$  chain polypeptide noncovalently associates with a single  $\beta$  chain. There are now about 14 distinct  $\alpha$  chain polypeptides and at least about 8 different  $\beta$  chain polypeptides which constitute the integrin family of cell adhesion receptors. In general, different binding specificities and tissue distributions are derived from unique combinations of the  $\alpha$  and  $\beta$  chain polypeptides or integrin subunits. The family to which a particular integrin is associated with is usually characterized by the  $\beta$  subunit. However, the ligand binding activity of the integrin is largely influenced by the  $\alpha$  subunit. For example, vitronectin binding integrins contain the  $\alpha_v$  integrin subunit.

It is now known that the vitronectin binding integrins consist of at least three different  $\alpha_v$  containing integrins. These  $\alpha_v$  containing integrins include  $\alpha_v\beta_3$ ,  $\alpha_v\beta_1$  and  $\alpha_v\beta_5$ , all of which exhibit different ligand binding specificities. For example, in addition to vitronectin,  $\alpha_v\beta_3$  binds to a large variety of

5 extracellular matrix proteins including fibronectin,  
fibrinogen, laminin, thrombospondin, von Willebrand  
10 factor, collagen, osteopontin and bone sialoprotein I.  
The integrin  $\alpha_v\beta_1$  binds to fibronectin, osteopontin and  
5 vitronectin whereas  $\alpha_v\beta_3$  is known to bind to vitronectin  
and osteopontin.

15 As cell adhesion receptors, integrins are  
involved in a variety of physiological processes  
including, for example, cell attachment, cell migration  
20 and cell proliferation. Different integrins play  
different roles in each of these biological processes and  
the inappropriate regulation of their function or  
activity can lead to various pathological conditions.  
For example, inappropriate endothelial cell proliferation  
25 during neovascularization of a tumor has been found to be  
mediated by cells expressing vitronectin binding  
integrins. In this regard, the inhibition of the  
vitronectin-binding integrin  $\alpha_v\beta_3$  also inhibits this  
30 process of tumor neovascularization. By this same  
criteria,  $\alpha_v\beta_3$  has also been shown to mediate the abnormal  
cell proliferation associated with restenosis and  
granulation tissue development in cutaneous wounds, for  
35 example. Additional diseases or pathological states  
mediated or influenced by  $\alpha_v\beta_3$  include, for example,  
25 metastasis, osteoporosis, age-related macular  
degeneration and diabetic retinopathy, and inflammatory  
40 diseases such as rheumatoid arthritis and psoriasis.  
Thus, agents which can specifically inhibit vitronectin-  
binding integrins would be valuable for the therapeutic  
45 30 treatment of diseases.

Many integrins mediate their cell adhesive  
functions by recognizing the tripeptide sequence Arg-Gly-  
50 Asp (RGD) found within a large number of extracellular

5 matrix proteins. A variety of approaches have attempted  
to model agents after this sequence to target a  
particular integrin-mediated pathology. Such approaches  
10 include, for example, the use of RGD-containing peptides  
5 and peptide analogues which rely on specificity to be  
conferred by the sequences flanking the RGD core  
tripeptide sequence. Although there has been some  
15 limited success, most RGD-based inhibitors have been  
shown to be, at most, selective for the targeted integrin  
20 and therefore exhibit some cross-reactivity to other  
non-targeted integrins. Such cross-reactive inhibitors  
therefore lack the specificity required for use as an  
25 efficacious therapeutic. This is particularly true for  
previously identified inhibitors of the integrin  $\alpha_v\beta_3$ .

25 15. Monoclonal antibodies on the other hand exhibit  
the specificity required to be used as an effective  
therapeutic. Antibodies also have the advantage in that  
30 they can be routinely generated against essentially any  
desired antigen. Moreover, with the development of  
20 combinatorial libraries, antibodies can now be produced  
faster and more efficiently than by previously used  
35 methods within the art. The use of combinatorial  
methodology also allows for the selection of the desired  
antibody along with the simultaneous isolation of the  
25 encoding heavy and light chain nucleic acids. Thus,  
40 further modification can be performed to the  
combinatorial antibody without the incorporation of an  
additional cloning step.

45 Regardless of the potential advantages  
30 associated with the use of monoclonal antibodies as  
therapeutics, these molecules nevertheless have the  
drawback in that they are almost exclusively derived from  
50 non-human mammalian organisms. Therefore, their use as

therapeutics is limited by the fact that they will normally elicit a host immune response. Methods for substituting the antigen binding site or complementarity determining regions (CDRs) of the non-human antibody into a human framework have been described. Such methods vary in terms of which amino acid residues should be substituted as the CDR as well as which framework residues should be changed to maintain binding specificity. In this regard, it is understood that proper orientation of the  $\beta$  sheet architecture, correct packing of the heavy and light chain interface and appropriate conformation of the CDRs are all important for preserving antigen specificity and affinity within the grafted antibody. However, all of these methods require knowledge of the nucleotide and amino acid sequence of the non-human antibody and the availability of an appropriately modeled human framework.

Thus, there exists a need for the availability of nucleic acids encoding integrin inhibitory antibodies which can be used as compatible therapeutics in humans. For  $\alpha_v\beta_3$ -mediated diseases, the present invention satisfies this need and provides related advantages as well.

#### SUMMARY OF THE INVENTION

The invention provides enhanced LM609 grafted antibodies exhibiting selective binding affinity to  $\alpha_v\beta_3$ , or a functional fragment thereof. The invention also provides nucleic acid molecules encoding the enhanced LM609 grafted antibodies. Additionally provided are methods of inhibiting a function of  $\alpha_v\beta_3$  by contacting  $\alpha_v\beta_3$  with an enhanced LM609 grafted antibody.



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**BRIEF DESCRIPTION OF THE DRAWINGS**

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Figure 1 shows the nucleotide and deduced amino acid sequence of the variable region of the antibody Vitaxin. Figure 1A shows the nucleotide and deduced amino acid sequences for the Vitaxin heavy chain variable region (Gln1-Ser117; SEQ ID NOS:1 and 2, respectively) while Figure 1B shows the nucleotide and deduced amino acid sequences for the Vitaxin light chain variable region (Glu1-Lys107; SEQ ID NOS:3 and 4, respectively).

10 Figure 2 shows the nucleotide and deduced amino acid sequence of the variable region of the monoclonal antibody LM609. Figure 2A shows the nucleotide and deduced amino acid sequence of the LM609 heavy chain variable region (SEQ ID NOS:5 and 6, respectively). The variable region extends from amino acid Glu1 to Ala117. Figure 2B shows the nucleotide and deduced amino acid sequence of the LM609 light chain variable region (SEQ ID NOS:7 and 8, respectively). The variable region of the light chain extends from amino acid Asp1 to Lys107.

20 Figure 3 shows the competitive inhibition of LM609 IgG binding to the integrin  $\alpha_v\beta_3$  with recombinant LM609 Fab. Soluble recombinant murine LM609 Fab fragments were prepared from periplasmic fractions of M13 bacteriophage clones muLM609M13 12 and muLM609M13 29. The periplasm samples were serially diluted, mixed with either 1 ng/ml, 5 ng/ml, or 50 ng/ml of LM609 IgG and then incubated in 96 well plates coated with purified  $\alpha_v\beta_3$ . Plates were washed and bound LM609 IgG detected with goat anti-murine Fc specific antibody conjugated to alkaline phosphatase. Fab produced by clone muLM609M13 12 inhibits both 1 ng/ml and 5 ng/ml LM609 IgG binding at all concentrations of Fab greater than 1:27 dilution.

Figure 4 shows the characterization of Vitaxin binding specificity. Figure 4A shows specific binding of Vitaxin to the integrin  $\alpha_v\beta_3$ , compared to integrins  $\alpha_{IIb}\beta_3$  and  $\alpha_v\beta_5$ . Figure 4B shows the competitive inhibition of LM609 binding to  $\alpha_v\beta_3$  by Vitaxin. Figure 4C shows the competitive inhibition of fibrinogen binding to  $\alpha_v\beta_3$  by Vitaxin.

Figure 5 shows the inhibition of  $\alpha_v\beta_3$ -mediated cell attachment (5A) and migration (5B) by Vitaxin.

Figure 6 shows the reduction in tumor growth due to Vitaxin mediated inhibition of neovascularization. Figure 6A shows the inhibition of the  $\alpha_v\beta_3$ -negative Fg and HEp-3 human tumor fragments grown on chick chorioallantoic membranes (CAMs) following Vitaxin treatment. Figure 6B shows the growth inhibition of Vx2 carcinomas implanted subcutaneously in rabbits at two different Vitaxin doses administered 1 day post implantation. Figure 6C similarly shows Vx2 tumor growth inhibition as in Figure 6B, except that four different Vitaxin doses were administered beginning at 7 days post implantation.

Figure 7 shows the nucleotide and deduced amino acid sequence of the light chain variable region of the LM609 grafted antibody fragment (Glu1-Lys107; SEQ ID NOS:31 and 32, respectively). Position 49 of the light chain variable region can at least be either Arg or Met. The nucleotide and deduced amino acid sequence of the heavy chain variable region of the LM609 grafted antibody fragment is shown in Figure 1A (SEQ ID NOS:1 and 2, respectively).

Figure 8 shows the titration of LM609 grafted antibody variants and LM609 grafted Fab on immobilized  $\alpha_v\beta_3$ . Bacterial cell lysates containing LM609 grafted antibody (closed circles), LM609 grafted antibody variants with improved affinity isolated from the primary libraries (S102, closed squares; Y100, open squares; and Y101, open triangles) or from combinatorial libraries (closed triangles), or an irrelevant Fab (open circles) were titrated on immobilized  $\alpha_v\beta_3$ .

Figure 9 shows the construction of combinatorial libraries of enhanced LM609 grafted antibody variants containing multiple amino acid substitutions.

Figure 10 shows the inhibition of fibrinogen binding to  $\alpha_v\beta_3$  by LM609 grafted antibody variants. Figure 10A shows inhibition of fibrinogen binding to immobilized  $\alpha_v\beta_3$ . Figure 10B shows correlation of affinity of antibody variants with inhibition of fibrinogen binding.

Figure 11 shows the inhibition of M21 human melanoma cell adhesion to fibrinogen by LM609 grafted antibody variants. Cell binding to 10  $\mu\text{g/ml}$  fibrinogen-coated substrate was assessed in the presence of various concentrations of LM609 grafted Fab (closed triangles) or the enhanced LM609 grafted Fabs S102 (open circles), G102 (closed circles), or C37 (open triangles).

#### DETAILED DESCRIPTION OF THE INVENTION

The invention is directed to nucleic acids encoding the monoclonal antibody (MAb) LM609. This antibody specifically recognizes the integrin  $\alpha_v\beta_3$  and

5 inhibits its functional activity. The invention is also  
directed to nucleic acids encoding and to polypeptides  
comprising non-murine grafted forms of LM609. These  
10 grafted antibodies retain the binding specificity and  
5 inhibitory activity of the parent murine antibody LM609.  
The invention is additionally directed to optimized forms  
of LM609 grafted antibodies that exhibit increased  
15 binding affinity and specificity compared to the  
non-mouse parental forms of the LM609 grafted antibody.

20 In one embodiment, the hybridoma expressing  
LM609 was used as a source to generate and clone cDNAs  
encoding LM609. The heavy and light chain encoding cDNAs  
were sequenced and their CDR regions were substituted  
25 into a human antibody framework to generate the  
15 non-murine form of the antibody. The substitution or  
grafting of the CDRs was performed by codon-based  
mutagenesis to generate a combinatorial antibody Fab  
30 library consisting of members that presented alternative  
residues at certain positions. Screening of the library  
20 resulted in the isolation of Vitaxin. As a grafted  
antibody containing human framework sequences, it is  
35 unlikely that Vitaxin will elicit a host immune response  
and can therefore be advantageously used for the  
treatment of  $\alpha_v\beta_3$ -mediated diseases.

40 25 As used herein, the term "monoclonal antibody  
LM609" or "LM609" is intended to mean the murine  
monoclonal antibody specific for the integrin  $\alpha_v\beta_3$ , which  
is described by Cheresh, D.A. Proc. Natl. Acad. Sci. USA  
45 84:6471-6475 (1987) and by Cheresh and Spiro J. Biol.  
30 Chem. 262:17703-17711 (1987). LM609 was produced against  
and is reactive with the M21 cell adhesion receptor now  
known as the integrin  $\alpha_v\beta_3$ . LM609 inhibits the attachment  
50 of M21 cells to  $\alpha_v\beta_3$  ligands such as vitronectin,

fibrinogen and von Willebrand factor (Cheresh and Spiro, *supra*) and is also an inhibitor of  $\alpha_v\beta_3$ -mediated pathologies such as tumor induced angiogenesis (Brooks et al. Cell 79:1157-1164 (1994), granulation tissue development in cutaneous wound (Clark et al., Am. J. Pathology, 148:1407-1421 (1996)) and smooth muscle cell migration such as that occurring during restenosis (Choi et al., J. Vascular Surg., 19:125-134 (1994); Jones et al., Proc. Natl. Acad. Sci. 93:2482-2487 (1996)).

As used herein, the term "Vitaxin" is intended to refer to a non-mouse antibody or functional fragment thereof having substantially the same heavy and light chain CDR amino acid sequences as found in LM609. The term "Vitaxin" when used in reference to heavy or light chain polypeptides is intended to refer to a non-mouse heavy or light chain or functional fragment thereof having substantially the same heavy or light chain CDR amino acid sequences as found in the heavy or light chain of LM609, respectively. When used in reference to a functional fragment, not all LM609 CDRs need to be represented. Rather, only those CDRs that would normally be present in the antibody portion that corresponds to the functional fragment are intended to be referenced as the LM609 CDR amino acid sequences in the Vitaxin functional fragment. Similarly, the use of the term "Vitaxin" in reference to an encoding nucleic acid is intended to refer to a nucleic acid encoding a non-mouse antibody or functional fragment having substantially the same nucleotide sequence as the heavy and light chain CDR nucleotide sequences and encoding substantially the same CDR amino acid sequences as found in LM609.

As used herein, the term "LM609 grafted antibody" is intended to refer to a non-mouse antibody or functional fragment thereof having substantially the same heavy and light chain CDR amino acid sequences as found in LM609 and absent of the substitution of LM609 amino acid residues outside of the CDRs as defined by Kabat et al., U.S. Dept. of Health and Human Services, "Sequences of Proteins of Immunological Interest" (1983). The term "LM609 grafted antibody" or "LM609 grafted" when used in reference to heavy or light chain polypeptides is intended to refer to a non-mouse heavy or light chain or functional fragment thereof having substantially the same heavy or light chain CDR amino acid sequences as found in the heavy or light chain of LM609, respectively, and also absent of the substitution of LM609 residues outside of the CDRs as defined by Kabat et al., *supra*. When used in reference to a functional fragment, not all LM609 CDRs need to be represented. Rather, only those CDRs that would normally be present in the antibody portion that corresponds to the functional fragment are intended to be referenced as the LM609 CDR amino acid sequences in the LM609 grafted functional fragment. Similarly, the term "LM609 grafted antibody" or "LM609 grafted" used in reference to an encoding nucleic acid is intended to refer to a nucleic acid encoding a non-mouse antibody or functional fragment being absent of the substitution of LM609 amino acids outside of the CDRs as defined by Kabat et al., *supra* and having substantially the same nucleotide sequence as the heavy and light chain CDR nucleotide sequences and encoding substantially the same CDR amino acid sequences as found in LM609 and as defined by Kabat et al., *supra*.

5                   The term "grafted antibody" or "grafted" when  
used in reference to heavy or light chain polypeptides or  
functional fragments thereof is intended to refer to a  
10                   heavy or light chain or functional fragment thereof  
5                   having substantially the same heavy or light chain CDR of  
a donor antibody, respectively, and also absent of the  
substitution of donor amino acid residues outside of the  
15                   CDRs as defined by Kabat et al., *supra*. When used in  
reference to a functional fragment, not all donor CDRs  
20                   need to be represented. Rather, only those CDRs that  
would normally be present in the antibody portion that  
corresponds to the functional fragment are intended to be  
referenced as the donor CDR amino acid sequences in the  
functional fragment. Similarly, the term "grafted  
25                   antibody" or "grafted" when used in reference to an  
encoding nucleic acid is intended to refer to a nucleic  
acid encoding an antibody or functional fragment, being  
absent of the substitution of donor amino acids outside  
30                   of the CDRs as defined by Kabat et al., *supra* and having  
20                   substantially the same nucleotide sequence as the heavy  
and light chain CDR nucleotide sequences and encoding  
substantially the same CDR amino acid sequences as found  
35                   in the donor antibody and as defined by Kabat et al.,  
*supra*.

25                   The meaning of the above terms are intended to  
include minor variations and modifications of the  
40                   antibody so long as its function remains uncompromised.  
Functional fragments such as Fab, F(ab)<sub>2</sub>, Fv, single chain  
Fv (scFv) and the like are similarly included within the  
45                   definition of the terms LM609 and Vitaxin. Such  
functional fragments are well known to those skilled in  
the art. Accordingly, the use of these terms in  
describing functional fragments of LM609 or the Vitaxin  
50                   antibody are intended to correspond to the definitions

5 well known to those skilled in the art. Such terms are  
described in, for example, Harlow and Lane, Antibodies: A  
10 Laboratory Manual, Cold Spring Harbor Laboratory, New  
York (1989); Molec. Biology and Biotechnology: A  
5 Comprehensive Desk Reference (Myers, R.A. (ed.), New  
York: VCH Publisher, Inc.); Huston et al., Cell  
Biophysics, 22:189-224 (1993); Plückthun and Skerra,  
15 Meth. Enzymol., 178:497-515 (1989) and in Day, E.D.,  
Advanced Immunochemistry, Second Ed., Wiley-Liss, Inc.,  
10 New York, NY (1990).

20 As with the above terms used for describing  
functional fragments of LM609, Vitaxin and a LM609  
grafted antibody, the use of terms which reference other  
25 LM609, Vitaxin or LM609 grafted antibody domains,  
15 functional fragments, regions, nucleotide and amino acid  
sequences and polypeptides or peptides, is similarly  
intended to fall within the scope of the meaning of each  
30 term as it is known and used within the art. Such terms  
include, for example, "heavy chain polypeptide" or "heavy  
20 chain", "light chain polypeptide" or "light chain",  
"heavy chain variable region" ( $V_H$ ) and "light chain  
35 variable region" ( $V_L$ ) as well as the term "complementarity  
determining region" (CDR).

40 In the case where there are two or more  
25 definitions of a term which is used and/or accepted  
within the art, the definition of the term as used herein  
is intended to include all such meanings unless  
45 explicitly stated to the contrary. A specific example is  
the use of the term "CDR" to describe the non-contiguous  
30 antigen combining sites found within the variable region  
of both heavy and light chain polypeptides. This  
particular region has been described by Kabat et al.,  
50 *supra*, and by Chothia et al., J. Mol. Biol. 196:901-917



(1987) and by MacCallum et al., J. Mol. Biol. 262:732-745 (1996) where the definitions include overlapping or subsets of amino acid residues when compared against each other. Nevertheless, application of either definition to refer to a CDR of LM609, Vitaxin, LM609 grafted antibodies or variants thereof is intended to be within the scope of the term as defined and used herein. The amino acid residues which encompass the CDRs as defined by each of the above cited references are set forth below in Table 1 as a comparison.

Table 1: CDR Definitions

	<u>Kabat<sup>1</sup></u>	<u>Chothia<sup>2</sup></u>	<u>MacCallum<sup>3</sup></u>
V <sub>H</sub> CDR1	31-35	26-32	30-35
V <sub>H</sub> CDR2	50-65	53-55	47-58
V <sub>H</sub> CDR3	95-102	96-101	93-101
V <sub>L</sub> CDR1	24-34	26-32	30-36
V <sub>L</sub> CDR2	50-56	50-52	46-55
V <sub>L</sub> CDR3	89-97	91-96	89-96

<sup>1</sup> Residue numbering follows the nomenclature of Kabat et al., *supra*

<sup>2</sup> Residue numbering follows the nomenclature of Chothia et al., *supra*

<sup>3</sup> Residue numbering follows the nomenclature of MacCallum et al., *supra*

As used herein, the term "substantially" or "substantially the same" when used in reference to a nucleotide or amino acid sequence is intended to mean that the nucleotide or amino acid sequence shows a considerable degree, amount or extent of sequence identity when compared to a reference sequence. Such considerable degree, amount or extent of sequence

identity is further considered to be significant and meaningful and therefore exhibit characteristics which are definitively recognizable or known. Thus, a nucleotide sequence which is substantially the same nucleotide sequence as a heavy or light chain of LM609, Vitaxin, or a LM609 grafted antibody including fragments thereof, refers to a sequence which exhibits characteristics that are definitively known or recognizable as encoding or as being the amino acid sequence of LM609, Vitaxin or a LM609 grafted antibody. Minor modifications thereof are included so long as they are recognizable as a LM609, Vitaxin or a LM609 grafted antibody sequence. Similarly, an amino acid sequence which is substantially the same amino acid sequence as a heavy or light chain of Vitaxin, a LM609 grafted antibody or functional fragment thereof, refers to a sequence which exhibits characteristics that are definitively known or recognizable as representing the amino acid sequence of Vitaxin or a LM609 grafted antibody and minor modifications thereof.

When determining whether a nucleotide or amino acid sequence is substantially the same as Vitaxin or a LM609 grafted antibody, consideration is given to the number of changes relative to the Vitaxin or LM609 grafted antibody together with whether the function is maintained. For example, a single amino acid change in a 3 amino acid CDR or several changes in a 16 amino acid CDR are considered to be substantially the same if  $\alpha_v\beta_3$  binding function is maintained. Thus, a nucleotide or amino acid sequence is substantially the same if it exhibits characteristics that are definitively known or recognizable as representing the nucleotide or amino acid sequence of Vitaxin or a LM609 grafted antibody and minor

modifications thereof as long as Vitaxin or LM609 grafted antibody function is maintained.

As used herein, the term "fragment" when used in reference to a nucleic acid encoding LM609, Vitaxin or a LM609 grafted antibody is intended to mean a nucleic acid having substantially the same sequence as a portion of a nucleic acid encoding LM609, Vitaxin or a LM609 grafted antibody. The nucleic acid fragment is sufficient in length and sequence to selectively hybridize to an LM609, a Vitaxin or a LM609 grafted antibody encoding nucleic acid or a nucleotide sequence that is complementary to an LM609, Vitaxin or LM609 grafted antibody encoding nucleic acid. Therefore, fragment is intended to include primers for sequencing and polymerase chain reaction (PCR) as well as probes for nucleic acid blot or solution hybridization. The meaning of the term is also intended to include regions of nucleotide sequences that do not directly encode LM609 polypeptides such as the introns, and the untranslated region sequences of the LM609 encoding gene.

As used herein, the term "functional fragment" when used in reference to Vitaxin, to a LM609 grafted antibody or to heavy or light chain polypeptides thereof is intended to refer to a portion of Vitaxin or a LM609 grafted antibody including heavy or light chain polypeptides which still retains some or all of the  $\alpha_v\beta_3$  binding activity,  $\alpha_v\beta_3$  binding specificity and/or integrin  $\alpha_v\beta_3$ -inhibitory activity. Such functional fragments can include, for example, antibody functional fragments such as Fab, F(ab)<sub>2</sub>, Fv, single chain Fv (scFv). Other functional fragments can include, for example, heavy or light chain polypeptides, variable region polypeptides or CDR polypeptides or portions thereof so long as such

functional fragments retain binding activity, specificity or inhibitory activity. The term is also intended to include polypeptides encompassing, for example, modified forms of naturally occurring amino acids such as D-stereoisomers, non-naturally occurring amino acids, amino acid analogues and mimetics so long as such polypeptides retain functional activity as defined above.

As used herein, the term "enhanced" when used in reference to Vitaxin, a LM609 grafted antibody or a functional fragment thereof is intended to mean that a functional characteristic of the antibody has been altered or augmented compared to a reference antibody so that the antibody exhibits a desirable property or activity. An antibody exhibiting enhanced activity can exhibit, for example, higher affinity or lower affinity binding, or increased or decreased association or dissociation rates compared to a reference antibody. An antibody exhibiting enhanced activity can also exhibit increased stability such as increased half-life in a particular organism. For example, an antibody activity can be enhanced to increase stability by decreasing susceptibility to proteolysis. If enhanced activity such as higher affinity binding or increased stability is desired, mutations can be introduced into framework or CDR amino acid residues and the resulting antibody variants screened for higher affinity binding to  $\alpha_v\beta_3$ , or increased stability relative to a reference antibody such as the LM609 grafted parent antibody. An antibody exhibiting enhanced activity can also exhibit lower affinity binding, including decreased association rates or increased dissociation rates, if desired. An enhanced antibody exhibiting lower affinity binding is useful, for example, for penetrating a solid tumor. In contrast to a higher affinity antibody, which would bind to the

5 peripheral regions of the tumor but would be unable to  
penetrate to the inner regions of the tumor due to its  
10 high affinity, a lower affinity antibody would be  
advantageous for penetrating the inner regions of the  
5 tumor.

15 The invention provides a nucleic acid encoding  
a heavy chain polypeptide for Vitaxin or a functional  
fragment thereof. Also provided is a nucleic acid  
encoding a light chain polypeptide for Vitaxin or a  
20 functional fragment thereof. The nucleic acids consist  
of substantially the same heavy or light chain variable  
region nucleotide sequences as those shown in Figure 1A  
and 1B (SEQ ID NOS:1 and 3, respectively) or a fragment  
25 thereof.

15 Vitaxin, including functional fragments  
thereof, is a non-mouse antibody which exhibits  
30 substantially the same binding activity, binding  
specificity and inhibitory activity as LM609. The  
Vitaxin Fv Fragment was produced by functionally  
20 replacing CDRs within human heavy and light chain  
variable region polypeptides with the CDRs derived from  
35 LM609. Functional replacement of the CDRs was performed  
by recombinant methods known to those skilled in the art.  
Such methods are commonly referred to as CDR grafting and  
40 25 are the subject matter of U.S. Patent No. 5,225,539.  
Such methods can also be found described in "Protein  
Engineering of Antibody Molecules for Prophylactic and  
Therapeutic Applications in Man," Clark, M. (ed.),  
45 Nottingham, England: Academic Titles (1993).

30 Briefly, LM609 nucleic acid fragments having  
substantially the same nucleotide and encoding  
50 substantially the same amino acid sequence of each of the

5 heavy and light chain CDRs were synthesized and  
substituted into each of the respective human chain  
encoding nucleic acids. To maintain functionality of the  
10 newly derived Vitaxin antibody, modifications were  
5 performed within the non-CDR framework region. These  
individual changes were made by generating a population  
of CDR grafted heavy and light chain variable regions  
15 wherein all possible changes outside of the CDRs were  
represented and then selecting the appropriate antibody  
10 by screening the population for binding activity. This  
screen resulted in the selection of the Vitaxin antibody  
20 described herein.

The nucleotide sequences of the Vitaxin heavy  
and light chain variable regions are shown in Figures 1A  
25 and 1B, respectively. These sequences correspond  
substantially to those that encode the heavy and light  
chain variable region polypeptides of Vitaxin. These  
30 Vitaxin nucleic acids are intended to include both the  
sense and anti-sense strands of the Vitaxin encoding  
20 sequences. Single- and double-stranded nucleic acids are  
similarly included as well as non-coding portions of the  
35 nucleic acid such as introns, 5'- and 3'-untranslated  
regions and regulatory sequences of the gene for example.

As shown in Figure 1A, the Vitaxin heavy chain  
40 25 variable region polypeptide is encoded by a nucleic acid  
of about 351 nucleotides in length which begins at the  
amino terminal Gln1 residue of the variable region  
through to Ser117. This Vitaxin heavy chain variable  
45 region encoding nucleic acid is joined to a human IgG1  
30 constant region to yield a coding region of 1431  
nucleotides which encodes a heavy chain polypeptide of  
477 total amino acids. Shown in Figure 1B is the Vitaxin  
50 light chain variable region polypeptide which is encoded

5 by a nucleic acid of about 321 nucleotides in length  
beginning at the amino terminal Glu1 residue of the  
variable region through to Lys107. This Vitaxin light  
10 chain variable region nucleic acid is joined to a human  
5 kappa construct region to yield a coding region of 642  
nucleotides which code for a light chain polypeptide of  
214 total amino acids.

15  
Minor modification of these nucleotide  
sequences are intended to be included as heavy and light  
20 chain Vitaxin encoding nucleic acids and their functional  
fragments. Such minor modifications include, for  
example, those which do not change the encoded amino acid  
sequence due to the degeneracy of the genetic code as  
well as those which result in only a conservative  
25 substitution of the encoded amino acid sequence.  
Conservative substitutions of encoded amino acids  
include, for example, amino acids which belong within the  
following groups: (1) non-polar amino acids (Gly, Ala,  
30 Val, Leu, and Ile); (2) polar neutral amino acids (Cys,  
20 Met, Ser, Thr, Asn, and Gln); (3) polar acidic amino  
acids (Asp and Glu); (4) polar basic amino acids (Lys,  
35 Arg and His); and (5) aromatic amino acids (Phe, Trp,  
Tyr, and His). Other minor modifications are included  
within the nucleic acids encoding Vitaxin heavy and light  
25 chain polypeptides so long as the nucleic acid or encoded  
polypeptides retain some or all of their function as  
40 described herein.

45  
Thus, the invention also provides a nucleic  
acid encoding a Vitaxin heavy chain or functional  
30 fragment thereof wherein the nucleic acid encodes  
substantially the same heavy chain variable region amino  
acid sequence of Vitaxin as that shown in Figure 1A (SEQ  
50 ID NO:2) or a fragment thereof. Similarly, the invention

5 also provides a nucleic acid encoding a Vitaxin light  
chain or functional fragment thereof wherein the nucleic  
acid encodes substantially the same light chain variable  
10 region amino acid sequence of Vitaxin as that shown in  
5 Figure 1B (SEQ ID NO:4) or a fragment thereof.

15 In addition to conservative substitutions of  
amino acids, minor modifications of the Vitaxin encoding  
nucleotide sequences which allow for the functional  
10 replacement of amino acids are also intended to be  
included within the definition of the term. The  
20 substitution of functionally equivalent amino acids  
encoded by the Vitaxin nucleotide sequences is routine  
and can be accomplished by methods known to those skilled  
25 in the art. Briefly, the substitution of functionally  
equivalent amino acids can be made by identifying the  
amino acids which are desired to be changed,  
incorporating the changes into the encoding nucleic acid  
30 and then determining the function of the recombinantly  
20 expressed and modified Vitaxin polypeptide or  
polypeptides. Rapid methods for making and screening  
multiple simultaneous changes are well known within the  
35 art and can be used to produce a library of encoding  
nucleic acids which contain all possible or all desired  
25 changes and then expressing and screening the library for  
Vitaxin polypeptides which retain function. Such methods  
40 include, for example, codon based mutagenesis, random  
oligonucleotide synthesis and partially degenerate  
oligonucleotide synthesis.

45 30 Codon based mutagenesis is the subject matter  
of U.S. Patent Nos. 5,264,563 and 5,523,388 and is  
advantageous for the above procedures since it allows for  
the production of essentially any and all desired  
50 frequencies of encoded amino acid residues at any and all



5 particular codon positions within an oligonucleotide.  
Such desired frequencies include, for example, the truly  
random incorporation of all twenty amino acids or a  
10 specified subset thereof as well as the incorporation of  
5 a predetermined bias of one or more particular amino  
acids so as to incorporate a higher or lower frequency of  
the biased residues compared to other incorporated amino  
15 acid residues. Random oligonucleotide synthesis and  
partially degenerate oligonucleotide synthesis can  
20 similarly be used for producing and screening for  
functionally equivalent amino acid changes. However, due  
to the degeneracy of the genetic code, such methods will  
incorporate redundancies at a desired amino acid  
position. Random oligonucleotide synthesis is the  
25 coupling of all four nucleotides at each nucleotide  
position within a codon whereas partially degenerate  
oligonucleotide synthesis is the coupling of equal  
portions of all four nucleotides at the first two  
30 nucleotide positions, for example, and equal portions of  
20 two nucleotides at the third position. Both of these  
latter synthesis methods can be found described in, for  
example, Cwirla et al., Proc. Natl. Acad. Sci. USA  
35 87:6378-6382, (1990) and Devlin et al., Science 249:404-  
406, (1990).

25 Identification of amino acids to be changed can  
be accomplished by those skilled in the art using current  
40 information available regarding the structure and  
function of antibodies as well as available and current  
information encompassing methods for CDR grafting  
45 30 procedures. For example, CDRs can be identified within  
the donor antibody by any or all of the criteria  
specified in Kabat et al., *supra*, Chothia et al., *supra*,  
and/or MacCallum et al., *supra*, and any or all  
50 non-identical amino acid residues falling outside of

5 these CDR sequences can be changed to functionally  
equivalent amino acids. Using the above described  
10 methods known within the art, any or all of the  
non-identical amino acids can be changed either alone or  
5 in combination with amino acids at different positions to  
incorporate the desired number of amino acid  
substitutions at each of the desired positions. The  
15 Vitaxin polypeptides containing the desired substituted  
amino acids are then produced and screened for retention  
20 or augmentation of function compared to the unsubstituted  
Vitaxin polypeptides. Production of the substituted  
25 Vitaxin polypeptides can be accomplished by, for example,  
recombinant expression using methods known to those  
skilled in the art. Those Vitaxin polypeptides which  
15 exhibit retention or augmentation of function compared to  
unsubstituted Vitaxin are considered to contain minor  
25 modifications of the encoding nucleotide sequence which  
result in the functional replacement of one or more amino  
30 acids.

20 The functional replacement of amino acids is  
beneficial when producing grafted antibodies having human  
35 framework sequences since it allows for the rapid  
identification of equivalent amino acid residues without  
the need for structural information or the laborious  
25 procedures necessary to assess and identify which amino  
acid residues should be considered for substitution in  
40 order to successfully transfer binding function from the  
donor. Moreover, it eliminates the actual step-wise  
procedures to change and test the amino acids identified  
45 30 for substitution. Essentially, using the functional  
replacement approach described above, all non-identical  
amino acid residues between the donor and the human  
framework can be identified and substituted with any or  
50 all other possible amino acid residues at each

5 non-identical position to produce a population of  
substituted polypeptides containing all possible or all  
desired permutations and combinations. The population of  
10 substituted polypeptides can then be screened for those  
5 substituted polypeptides which retain function. Using  
the codon based mutagenesis procedures described above,  
the generation of a library of substituted amino acid  
15 residues and the screening of functionally replaced  
residues has been used for the rapid production of  
10 grafted therapeutic antibodies as well as for the rapid  
alteration of antibody affinity. Such procedures are  
20 exemplified in, for example, Rosok et al., J. Biol. Chem.  
271:22611-22618 (1996) and in Glaser et al., J. Immunol.  
149:3903-3913 (1992), respectively.

25 15 In addition to framework residues, amino acids  
in one or more CDRs can be functionally replaced to allow  
identification of a modified LM609 grafted antibody  
30 having enhanced activity. Using the methods described  
above for framework residues, amino acid substitutions  
20 can similarly be introduced into one or more CDRs in an  
LM609 grafted antibody. The modified LM609 grafted  
antibody can be tested for binding activity to determine  
35 whether  $\alpha_v\beta_3$  binding activity is maintained. The modified  
LM609 grafted antibody can be further tested to determine  
25 if activity has been enhanced. Functional replacement of  
amino acid residues in one or more CDRs therefore allows  
40 the identification of an enhanced LM609 grafted antibody  
having a desirable property such as enhanced activity.

45 To generate modified LM609 grafted antibodies  
30 and select those with enhanced activity, several  
approaches can be employed in the selection of the number  
of residues within a CDR to mutate as well as the number  
50 of CDRs within a LM609 grafted antibody to modify. The

choice of selection criteria for mutagenesis of CDRs will depend on the need and desired application of the enhanced antibody. For example, one or a few amino acid positions within a single CDR can be modified to contain selected amino acids at that position. Alternatively, the targeted amino acid positions can be modified to contain all possible amino acids at that position. The resultant population of modified antibodies can then be screened for enhanced activity.

The construction of modified LM609 grafted antibody populations can also be made where all amino acids positions within a CDR have been mutated to contain all possible amino acids and where amino acid positions within multiple CDRs have been modified to contain variant amino acid residues. In this way, populations can be constructed which range from 2 to  $>10^7$  unique members. The larger the population, the more efficient will be the selection of an enhanced LM609 grafted antibody since there will be a larger number of different antibodies within the population. However, a small population of modified LM609 grafted antibodies can be made and successfully used for the selection of enhanced LM609 grafted antibodies. The size of the population of modified LM609 grafted antibodies will depend on the need of a particular application and can be determined by one skilled in the art.

The generation of modified LM609 grafted antibodies can be achieved by introducing amino acid substitutions into one or more CDRs of an LM609 grafted antibody. For example, single amino acid substitutions can be systematically introduced into a CDR by changing a given amino acid in the CDR to any or all amino acids. Amino acid substitutions can also be introduced into all

5 amino acid positions in one or more of the CDRs or in all  
of the CDRs, generating a population of modified LM609  
grafted antibody variants. This population of modified  
10 LM609 grafted antibody variants having single amino acid  
5 substitutions can be screened to identify those variants  
that maintain  $\alpha_v\beta_3$  binding activity. The variants having  
 $\alpha_v\beta_3$  binding activity can be further characterized to  
15 identify those variants having enhanced activity. Such a  
systematic approach to introducing single amino acid  
20 substitutions and generating a population of LM609  
grafted antibody variants to screen for enhanced LM609  
grafted antibodies having high affinity binding to  $\alpha_v\beta_3$  is  
described in Example VI.

25 In addition to generating a population of  
15 modified LM609 grafted antibody variants, a particular  
CDR or a particular amino acid in a CDR can be selected  
to introduce one or more amino acid substitutions. For  
30 example, sequence homology or a structural model can be  
used to identify particular amino acid positions to  
20 introduce amino acid substitutions. In this example,  
only one or a few modified LM609 grafted antibody  
35 variants are generated and screened for binding activity  
to  $\alpha_v\beta_3$ . One of skill in the art will know or can  
determine whether it is desirable to generate a large  
25 population of modified LM609 grafted antibody variants or  
40 to generate a limited number of modified LM609 grafted  
antibody variants to screen and identify an enhanced  
LM609 grafted antibody having enhanced activity.

45 In addition to identifying enhanced LM609  
30 grafted antibodies by generating a population of modified  
LM609 grafted antibodies having single amino acid  
substitutions in a CDR and screening for enhanced  
50 activity, enhanced LM609 grafted antibody variants can

5 also be generated by combining two or more mutations,  
each known to independently result in enhanced activity,  
into a single antibody. When there are more than two  
10 mutations, an efficient way to identify combinations of  
5 mutations which further augment activity is to construct  
all possible combinations and permutations and then  
select for those with enhanced activity. For example,  
15 two single mutations in one or more CDRs can be combined  
to generate a new modified LM609 grafted antibody having  
10 two CDR mutations and screened to determine if the  $\alpha_v\beta_3$   
binding activity is increased over that of the single  
20 mutants. Similarly, three mutations can be combined and  
the resulting modified LM609 grafted antibody screened  
for enhanced binding activity. Using such an approach of  
15 combining CDR mutations, a new population of modified  
25 LM609 grafted antibody variants can be generated by  
incorporating all combinations of the single CDR  
mutations resulting in enhanced activity into new  
30 modified LM609 grafted antibody variants and screening to  
20 obtain an optimized enhanced LM609 grafted antibody.

An iterative, step-wise approach to identifying  
35 an enhanced LM609 grafted antibody is advantageous in  
that it allows the identification of an antibody having  
optimal binding activity without the need to generate and  
25 screen a large number of modified LM609 grafted antibody  
40 variants. For example, using the approach described in  
Examples VI and VII in which single mutants were  
identified and combined into a new population of LM609  
grafted antibody variants, enhanced LM609 grafted  
45 30 antibodies having higher affinity were identified by  
generating 2592 unique variants. In contrast, complete  
randomization of a single eight amino acid residue CDR  
would require  $>10^{10}$  unique variants. Therefore, such an  
50 iterative approach allows identification of enhanced

5 LM609 grafted antibodies having enhanced activity such as  
high affinity binding by generating a relatively small  
number of unique modified LM609 grafted antibody variants  
10 and screening and identifying those enhanced LM609  
5 grafted antibody variants exhibiting high affinity  
binding.

15 An iterative, step-wise approach to identifying  
enhanced LM609 variants can also be performed using  
additional steps. Instead of generating all combinations  
10 of single amino acid mutations, the single amino acid  
20 mutations can be combined in pairs to generate all  
combinations of double mutants and screened for activity.  
Those double mutants having enhanced activity can be  
combined with any or all single mutants to generate  
25 triple mutants that are screened for enhanced binding  
activity. Each iterative round of generating modified  
LM609 grafted antibody variants can incorporate  
30 additional single mutations, and the resulting modified  
LM609 grafted antibodies can be screened for enhanced  
20 activity. The step-wise generation of LM609 grafted  
antibody variants can thus be used to identify an  
35 optimized LM609 grafted antibody. Additionally, such an  
iterative approach also allows for the identification of  
numerous enhanced antibodies which exhibit a range of  
25 different, enhanced binding activities.

40 An optimized LM609 grafted antibody can also be  
referred to as an LM609-like grafted antibody or an  
 $\alpha\beta$ -specific grafted antibody and is recognizable because  
45 the antibody or functional fragments thereof retains the  
functional characteristics of LM609. For example,  
30 enhanced LM609 grafted antibody variants, which have a  
single amino acid substitution and have enhanced  
50 activity, can be identified and correlated with a

5 specific amino acid substitution. These amino acid  
substitutions can be combined to generate a new modified  
LM609 grafted antibody that is tested for activity. Such  
10 a combination of advantageous CDR amino acid  
5 substitutions can result in an optimized LM609 grafted  
antibody with multiple CDRs having at least one amino  
acid substitution or a single CDR having multiple amino  
15 acid substitutions, where the modified LM609 grafted  
antibody has enhanced activity.

10 Enhanced LM609 grafted antibodies, particularly  
20 those optimized by functional replacement of amino acid  
residues in the CDRs, have desirable enhanced properties  
such as increased affinity. For example, an optimized  
LM609 grafted antibody having increased affinity will  
25 have higher affinity than the parent antibody used for  
introducing functional replacement of amino acids.  
Higher affinity is determined relative to a reference  
antibody having a similar structure. For example, if the  
30 optimized LM609 grafted antibody is an intact antibody  
20 containing two heavy chains and two light chains, then  
higher affinity is determined relative to the intact  
parent LM609 grafted antibody. Similarly, if the  
35 optimized LM609 grafted antibody is an Fab, then higher  
affinity is determined relative to the Fab of the parent  
25 LM609 grafted antibody.

40 Although it is not necessary to proceed through  
multiple optimization steps to obtain a high affinity  
LM609 grafted antibody, in general, the increase in  
45 affinity can correlate with the number of modifications  
30 within and between CDRs as well as with the number of  
optimization steps. Therefore, LM609 grafted antibodies  
will exhibit a variety of ranges. For example, LM609  
50 grafted antibodies having enhanced affinity will have up



5 to about 2-fold higher affinity or greater, generally  
greater than about 2- to 5-fold higher affinity such as  
greater than about 4- to 5-fold higher affinity or about  
10 5- to 10-fold higher affinity than the reference  
5 antibody. Particularly, a LM609 grafted antibody having  
enhanced affinity will have greater than about 10- to  
50-fold higher affinity, greater than about 50-fold  
15 higher affinity, or greater than about 100-fold higher  
affinity than the reference antibody.

10 As described above, functional replacement of  
20 CDR amino acid residues can be used to identify LM609  
grafted antibodies exhibiting higher affinity than a  
parent LM609 grafted antibody. Methods discussed above  
or below for introducing minor modifications into Vitaxin  
25 or LM609 grafted antibody encoding nucleotide sequences  
can similarly be used to generate a library of modified  
LM609 grafted antibody variants, including methods such  
as codon based mutagenesis, random oligonucleotide  
30 synthesis and partially degenerate oligonucleotide  
20 synthesis. For example, codon based mutagenesis has been  
used to generate such a library of modified LM609 grafted  
antibody variants having single amino acid substitutions  
35 (see Example VI).

40 After generating a library of modified LM609  
25 grafted antibody variants, the variants can be expressed  
and screened for binding activity to  $\alpha_v\beta_3$ . Methods well  
known to those skilled in the art related to determining  
antibody-antigen interactions are used to screen for  
45 modified LM609 grafted antibodies exhibiting binding  
30 activity to  $\alpha_v\beta_3$  (Harlow and Lane, *supra*). For example,  
an ELISA method has been used to screen a library of  
modified LM609 grafted antibody variants to identify  
50 those variants that maintained  $\alpha_v\beta_3$  binding activity (see

Example VI). Only those modified LM609 grafted antibodies that maintain  $\alpha_v\beta_3$  binding activity are considered for further characterization.

Modified LM609 grafted antibodies having  $\alpha_v\beta_3$  binding activity can be further characterized to determine which modified LM609 grafted antibody has enhanced activity. The type of assay used to assess enhanced activity depends on the particular desired characteristic. For example, if altered binding activity is desired, then binding assays that allow determination of binding affinity are used. Such assays include binding assays, competition binding assays and surface plasmon resonance as described in Example VI.

Introduction of single amino acid substitutions into CDRs of LM609 grafted antibodies can be used to generate a library of modified LM609 grafted antibodies and screen for binding activity to  $\alpha_v\beta_3$ . Those modified LM609 grafted antibodies exhibiting binding activity to  $\alpha_v\beta_3$  can then be further characterized to identify enhanced LM609 grafted antibodies exhibiting enhanced activity such as higher binding affinity. For example, using such an approach, a number of enhanced LM609 grafted antibodies having single amino acid substitutions were generated using the heavy chain variable region shown in Figure 1a (SEQ ID NO:2) and the light chain variable region shown in Figure 7 (SEQ ID NO:32), and LM609 grafted antibodies were identified displaying 2 to 13-fold improved affinity over the parent LM609 grafted antibody (see Example VI).

Following identification of enhanced LM609 grafted antibodies having a single amino acid substitution, the amino acid mutations can be combined to

5 further enhance activity. Methods discussed above for  
introducing single amino acid substitutions into CDRs can  
10 similarly be applied to combine amino acid substitutions.  
For example, a combinatorial library of amino acid  
5 mutations that resulted in enhanced  $\alpha_v\beta_3$  binding affinity  
was generated using degenerate oligonucleotides and two  
site hybridization mutagenesis as described in Example  
15 VII. Enhanced LM609 grafted antibodies containing  
multiple CDR amino acid substitutions were generated  
20 using the heavy chain variable region shown in Figure 1a  
(SEQ ID NO:2) and the light chain variable region shown  
in Figure 7 (SEQ ID NO:32), and LM609 grafted antibodies  
25 were identified having 20-fold higher affinity to greater  
than 90-fold higher affinity than the parent LM609  
grafted antibody.

In addition to combining CDR amino acid  
substitutions to generate an enhanced or optimized LM609  
30 grafted antibody, CDR amino acid substitutions can also  
be combined with framework mutations that contribute  
20 desirable properties to a LM609 grafted antibody. Thus,  
mutations in CDR or framework regions that enhance  
35 activity can be combined to further optimize LM609  
grafted antibodies.

The invention further provides fragments of  
40 25 Vitaxin heavy and light chain encoding nucleic acids  
wherein such fragments consist substantially of the same  
nucleotide or amino acid sequence as the variable region  
of Vitaxin heavy or light chain polypeptides. The  
45 variable region of the Vitaxin heavy chain polypeptide  
30 consists essentially of nucleotides 1-351 and of amino  
acid residues Gln1 to Ser117 of Figure 1A (SEQ ID NOS:1  
and 2, respectively). The variable region of the Vitaxin  
50 light chain polypeptide consists essentially of

nucleotides 1-321 and of amino acid residues Glu1 to Lys107 of Figure 1B (SEQ ID NOS:3 and 4, respectively). The termini of such variable region encoding nucleic acids is not critical so long as the intended purpose and function remains the same.

Fragments additional to the variable region nucleic acid fragments are provided as well. Such fragments include, for example, nucleic acids consisting substantially of the same nucleotide sequence as a CDR of a Vitaxin heavy or light chain polypeptide. Sequences corresponding to the Vitaxin CDRs include, for example, those regions defined by Kabat et al., *supra*, and/or those regions defined by Chothia et al., *supra*, as well as those defined by MacCallum et al., *supra*. The Vitaxin CDR fragments for each of the above definitions correspond to the nucleotides set forth below in Table 2. The nucleotide sequence numbering is taken from the primary sequence shown in Figures 1A and 1B (SEQ ID NOS:1 and 3) and conforms to the definitions previously set forth in Table 1.

**Table 2: Vitaxin CDR Nucleotide Residues**

	<u>Kabat</u>	<u>Chothia</u>	<u>MacCallum</u>
V <sub>H</sub> CDR1	91-105	76-96	88-105
V <sub>H</sub> CDR2	148-198	157-168	139-177
25 V <sub>H</sub> CDR3	295-318	298-315	289-315
V <sub>L</sub> CDR1	70-102	76-96	88-108
V <sub>L</sub> CDR2	148-168	148-156	136-165
45 V <sub>L</sub> CDR3	265-291	271-288	265-288

Similarly, the Vitaxin CDR fragments for each of the above definitions correspond to the amino acid residues set forth below in Table 3. The amino acid

residue number is taken from the primary sequence shown in Figures 1A and 1B (SEQ ID NOS:2 and 4) and conforms to the definitions previously set forth in Table 1.

**Table 3: Vitaxin CDR Amino Acid Residues**

		<u>Kabat</u>	<u>Chothia</u>	<u>MacCallum</u>
5	V <sub>H</sub> CDR1	Ser31-Ser35	Gly26-Tyr32	Ser30-Ser35
15	V <sub>H</sub> CDR2	Lys50-Gly66	Ser53-Gly56	Trp47-Tyr59
	V <sub>H</sub> CDR3	His99-Tyr106	Asn100-Ala105	Ala97-Ala105
	V <sub>L</sub> CDR1	Gln24-His34	Ser26-His32	Ser30-Tyr36
20	10 V <sub>L</sub> CDR2	Tyr50-Ser56	Tyr50-Ser52	Leu46-Ile55
	V <sub>L</sub> CDR3	Gln89-Thr97	Ser91-His96	Gln89-His96

Thus, the invention also provides nucleic acid fragments encoding substantially the same amino acid sequence as a CDR of a Vitaxin heavy or light chain polypeptide.

Nucleic acids encoding Vitaxin heavy and light chain polypeptides and fragments thereof are useful for a variety of diagnostic and therapeutic purposes. For example, the Vitaxin nucleic acids can be used to produce Vitaxin antibodies and functional fragments thereof having binding specificity and inhibitory activity against the integrin  $\alpha_v\beta_3$ . The antibody and functional fragments thereof can be used for the diagnosis or therapeutic treatment of  $\alpha_v\beta_3$ -mediated disease. Vitaxin and functional fragments thereof can be used, for example, to inhibit binding activity or other functional activities of  $\alpha_v\beta_3$  that are necessary for progression of an  $\alpha_v\beta_3$ -mediated disease. Other functional activities necessary for progression of  $\alpha_v\beta_3$ -mediated disease include, for example, the activation of  $\alpha_v\beta_3$ ,  $\alpha_v\beta_3$ -mediated signal transduction and the  $\alpha_v\beta_3$ -mediated prevention of

5 apoptosis. Advantageously, however, Vitaxin comprises  
non-mouse framework amino acid sequences and as such is  
less antigenic in regard to the induction of a host  
10 immune response. The Vitaxin nucleic acids of the  
5 inventions can also be used to model functional  
equivalents of the encoded heavy and light chain  
polypeptides.

15 Thus, the invention provides Vitaxin heavy  
chain and Vitaxin light chain polypeptides or functional  
10 fragments thereof. The Vitaxin heavy chain polypeptide  
exhibits substantially the same amino acid sequence as  
20 that shown in Figure 1A (SEQ ID NO:2) or functional  
fragment thereof whereas the Vitaxin light chain  
polypeptide exhibits substantially the same amino acid  
25 sequence as that shown in Figure 1B (SEQ ID NO:4) or  
functional fragment thereof. Also provided is a Vitaxin  
antibody or functional fragment thereof. The antibody is  
30 generated from the above heavy and light chain  
polypeptides or functional fragments thereof and exhibits  
20 selective binding affinity to  $\alpha, \beta_3$ .

35 The invention provides a nucleic acid encoding  
a heavy chain polypeptide for a LM609 grafted antibody.  
Also provided is a nucleic acid encoding a light chain  
polypeptide for a LM609 grafted antibody. The nucleic  
40 25 acids consist of substantially the same heavy chain  
variable region nucleotide sequence as that shown in  
Figure 1A (SEQ ID NO:1) and substantially the same light  
chain variable region nucleotide sequence as that shown  
45 in Figure 7 (SEQ ID NO:31) or a fragment thereof.

30 LM609 grafted antibodies, including functional  
fragments thereof, are non-mouse antibodies which exhibit  
50 substantially the same binding activity, binding

5 specificity and inhibitory activity as LM609. The LM609  
grafted antibody Fv fragments described herein are  
produced by functionally replacing the CDRs as defined by  
10 Kabat et al. , hereinafter referred to as "Kabat CDRs,"  
5 within human heavy and light chain variable region  
polypeptides with the Kabat CDRs derived from LM609.  
Functional replacement of the Kabat CDRs is performed by  
15 the CDR grafting methods previously described and which  
is the subject matter of U.S. Patent No. 5,225,539,  
10 *supra*. Substitution of amino acid residues outside of  
the Kabat CDRs can additionally be performed to maintain  
20 or augment beneficial binding properties so long as such  
amino acid substitutions do not correspond to a donor  
amino acid at that particular position. Such  
25 substitutions allow for the modulation of binding  
properties without imparting any mouse sequence  
characteristics onto the antibody outside of the Kabat  
CDRs. Although the production of such antibodies is  
30 described herein with reference to LM609 grafted  
20 antibodies, the substitution of such non-donor amino  
acids outside of the Kabat CDRs can be utilized for the  
production of essentially any grafted antibody. The  
35 production of LM609 grafted antibodies is described  
further below in Example V.

25 The nucleotide sequences of the LM609 grafted  
40 antibody heavy and light chain variable regions are shown  
in Figures 1A and 7, respectively. These sequences  
correspond substantially to those that encode the heavy  
and light chain variable region polypeptides of a LM609  
45 30 grafted antibody. These nucleic acids are intended to  
include both the sense and anti-sense strands of the  
LM609 grafted antibody encoding sequences. Single- and  
50 double-stranded nucleic acids are similarly included as  
well as non-coding portions of the nucleic acid such as

introns, 5'- and 3'-untranslated regions and regulatory sequences of the gene for example.

The nucleotide and amino acid residue boundaries for a LM609 grafted antibody are identical to those previously described for Vitaxin. For example, a LM609 grafted antibody heavy chain variable region polypeptide is encoded by a nucleic acid of about 351 nucleotides in length which begins at the amino terminal Gln1 residue of the variable region through to Ser117 (Figure 1A, SEQ ID NOS:1 and 2, respectively). The LM609 grafted antibody light chain variable region polypeptide is encoded by a nucleic acid of about 321 nucleotides in length beginning at the amino terminal Glu1 residue of the variable region through to Lys107 (Figure 7, SEQ ID NOS:31 and 32, respectively). As with Vitaxin, minor modification of these nucleotide sequences are intended to be included as heavy and light chain variable region encoding nucleic acids and their functional fragments.

Thus, the invention also provides a nucleic acid encoding a LM609 grafted antibody heavy chain wherein the nucleic acid encodes substantially the same heavy chain variable region amino acid sequence as that shown in Figure 1A (SEQ ID NO:2) or fragment thereof. Similarly, the invention also provides a nucleic acid encoding a LM609 grafted antibody light chain wherein the nucleic acid encodes substantially the same light chain variable region amino acid sequence as that shown in Figure 7 (SEQ ID NO:32) or fragment thereof.

In addition to conservative substitutions of amino acids, minor modifications of the LM609 grafted antibody encoding nucleotide sequences which allow for the functional replacement of amino acids are also



5 intended to be included within the definition of the  
term. Identification of amino acids to be changed can be  
accomplished by those skilled in the art using current  
10 information available regarding the structure and  
5 function of antibodies as well as available and current  
information encompassing methods for CDR grafting  
procedures. The substitution of functionally equivalent  
15 amino acids encoded by the LM609 grafted antibody  
nucleotide sequences is routine and can be accomplished  
10 by methods known to those skilled in the art. As  
described previously, such methods include, for example,  
20 codon based mutagenesis, random oligonucleotide synthesis  
and partially degenerate oligonucleotide synthesis and  
are beneficial when producing grafted antibodies since  
25 they allow for the rapid identification of equivalent  
amino acid residues without the need for structural  
information.

30 The invention further provides fragments of  
LM609 grafted antibody heavy and light chain encoding  
20 nucleic acids wherein such fragments consist  
substantially of the same nucleotide or amino acid  
sequence as the variable region of a LM609 grafted  
35 antibody heavy or light chain polypeptide. As with  
Vitaxin, the termini of such variable region encoding  
25 nucleic acids is not critical so long as the intended  
purpose and function remains the same.  
40

Fragments additional to the variable region  
nucleic acid fragments are provided as well and include,  
45 for example, nucleic acids consisting substantially of  
30 the same nucleotide sequence as a CDR of a LM609 grafted  
antibody heavy or light chain polypeptide. As with  
Vitaxin, sequences corresponding to the LM609 grafted  
50 antibody CDRs include, for example, those regions defined

5 by Kabat et al., *supra*, Chothia et al., *supra*, as well as  
those defined by MacCallum et al., *supra*. The LM609  
grafted antibody CDR regions will be similar to those  
10 described previously for Vitaxin. Moreover, such regions  
5 are well known and can be determined by those skilled in  
the art given the LM609 sequences and teachings provided  
herein. Thus, the invention also provides nucleic acid  
15 fragments encoding substantially the same amino acid  
sequence as a CDR of a LM609 grafted antibody heavy or  
10 light chain polypeptide.

20 As with Vitaxin, nucleic acids encoding LM609  
grafted antibody heavy and light chain polypeptides and  
fragments thereof are useful for a variety of diagnostic  
and therapeutic purposes. For example, LM609 grafted  
25 antibody encoding nucleic acids can be used to produce  
recombinant antibodies and functional fragments thereof  
having binding specificity and inhibitory activity  
against the integrin  $\alpha_v\beta_3$ . The antibody and functional  
30 fragments thereof can be used for the diagnosis or  
20 therapeutic treatment of  $\alpha_v\beta_3$ -mediated disease. Such  
diseases and methods of use for anti- $\alpha_v\beta_3$  antibodies have  
35 been described previously in reference to Vitaxin and are  
equally applicable to the LM609 grafted antibodies  
described herein.

40 25 Thus, the invention provides LM609 grafted  
antibody heavy chain and Vitaxin light chain polypeptides  
or functional fragments thereof. The LM609 grafted  
antibody heavy chain polypeptide exhibits substantially  
45 the same amino acid sequence as that shown in Figure 1A  
30 (SEQ ID NO:2) or functional fragment thereof whereas the  
LM609 grafted antibody light chain polypeptide exhibits  
substantially the same amino acid sequence as that shown  
50 in Figure 7 (SEQ ID NO:32). Also provided is a LM609

5 grafted antibody or functional fragment thereof. The  
antibody is generated from the above heavy and light  
chain polypeptides or functional fragments thereof and  
10 exhibits selective binding affinity to  $\alpha_v\beta_3$ .

5 The invention provides an enhanced LM609  
grafted antibody exhibiting selective binding affinity to  
15  $\alpha_v\beta_3$ . The enhanced LM609 grafted antibody contains at  
least one amino acid substitution in one or more CDRs of  
a LM609 grafted heavy chain variable region polypeptide  
10 or a LM609 grafted light chain variable region  
polypeptide, wherein the  $\alpha_v\beta_3$  binding affinity of the  
enhanced LM609 grafted antibody is maintained or  
enhanced.

25 To identify enhanced LM609 grafted antibodies,  
15 a library of modified LM609 grafted antibodies was  
generated as described above and in Example VI.  
Initially, LM609 CDRs were identified and selected to  
30 introduce single amino acid substitutions. Utilizing the  
numbering system of Kabat et al., *supra*, the CDR residues  
20 selected for mutagenesis were  $V_H$  CDR1 Gly-Phe-Thr-Phe-Ser-  
Ser-Tyr-Asp-Met-Ser (SEQ ID NO:34) (Gly<sup>26</sup>-Ser<sup>35</sup>);  $V_H$  CDR2  
35 Trp-Val-Ala-Lys-Val-Ser-Ser-Gly-Gly-Gly (SEQ ID NO:36)  
and Ser-Thr-Tyr-Tyr-Leu-Asp-Thr-Val-Gln-Gly (SEQ ID  
NO:38) (Trp<sup>47</sup>-Gly<sup>65</sup>);  $V_H$  CDR3 Ala-Arg-His-Asn-Tyr-Gly-Ser-  
40 Phe-Ala-Tyr (SEQ ID NO:40) (Ala<sup>93</sup>-Tyr<sup>102</sup>);  $V_L$  CDR1 Gln-Ala-  
Ser-Gln-Ser-Ile-Ser-Asn-His-Leu-His-Trp-Tyr (SEQ ID  
NO:42) (Gln<sup>24</sup>-Tyr<sup>36</sup>);  $V_L$  CDR2 Leu-Leu-Ile-Arg-Tyr-Arg-Ser-  
45 Gln-Ser-Ile-Ser (SEQ ID NO:44) (Leu<sup>46</sup>-Ser<sup>56</sup>); and  $V_L$  CDR3  
30 Gln-Gln-Ser-Gly-Ser-Trp-Pro-His-Thr (SEQ ID NO:46)  
(Gln<sup>89</sup>-Thr<sup>97</sup>).

5 The nucleotide sequences encoding the CDR  
residues selected for mutagenesis were V<sub>H</sub> CDR1 GGA TTC ACC  
TTC AGT AGC TAT GAC ATG TCT (SEQ ID NO:33); V<sub>H</sub> CDR2 TGG  
10 GTC GCA AAA GTT AGT AGT GGT GGT GGT (SEQ ID NO:35) and  
5 AGC ACC TAC TAT TTA GAC ACT GTG CAG GGC (SEQ ID NO:37); V<sub>H</sub>  
CDR3 GCA AGA CAT AAC TAC GGC AGT TTT GCT TAC (SEQ ID  
NO:39); V<sub>L</sub> CDR1 CAG GCC AGC CAA AGT ATT AGC AAC CAC CTA  
15 CAC TGG TAT (SEQ ID NO:41); V<sub>L</sub> CDR2 CTT CTC ATC CGT TAT  
CGT TCC CAG TCC ATC TCT (SEQ ID NO:43); and V<sub>L</sub> CDR3 CAA  
20 CAG AGT GGC AGC TGG CCT CAC ACG (SEQ ID NO:45).

20 Single amino acid substitutions can be  
introduced into the CDRs of an LM609 grafted antibody to  
generate a population of modified LM609 grafted  
25 antibodies. For example, every amino acid in one or more  
CDRs can be mutated to any or all amino acids to generate  
a population of modified LM609 grafted antibodies and the  
population screened for  $\alpha_v\beta_3$  binding activity. Although  
30 this population is generated by mutating amino acids in  
CDRs, populations can also be constructed where changes  
20 are made in the framework region residues or in both the  
CDRs and the framework. Such mutations in the variable  
35 regions can be made separately, in combination, or step-  
wise. Thus, the invention also provides for an enhanced  
LM609 grafted antibody, where the amino acid substitution  
25 is in the CDR or in the framework region.

40 The invention additionally provides an enhanced  
LM609 grafted antibody exhibiting enhanced binding  
affinity. Enhanced LM609 grafted antibodies exhibiting  
45 enhanced binding affinity include those containing at  
least one of the following CDRs having single amino acid  
30 substitutions:  
a V<sub>H</sub> CDR1 selected from the group consisting of Gly-Thr-  
50 Thr-Phe-Ser-Ser-Tyr-Asp-Met-Ser (SEQ ID NO:48), Gly-Phe-

5 Thr-Trp-Ser-Ser-Tyr-Asp-Met-Ser (SEQ ID NO:50) and Gly-  
Phe-Thr-Phe-Leu-Ser-Tyr-Asp-Met-Ser (SEQ ID NO:52);  
a V<sub>H</sub> CDR2 selected from the group consisting of Trp-Val-  
10 Ala-Lys-Val-Lys-Ser-Gly-Gly-Gly (SEQ ID NO:54), Ser-Thr-  
5 Tyr-Tyr-Pro-Asp-Thr-Val-Gln-Gly (SEQ ID NO:56) and Ser-  
Thr-Tyr-Tyr-Leu-Asp-Thr-Val-Glu-Gly (SEQ ID NO:58);  
a V<sub>H</sub> CDR3 selected from the group consisting of Ala-Arg-  
15 His-Asn-His-Gly-Ser-Phe-Ala-Tyr (SEQ ID NO:60), Ala-Arg-  
His-Asn-Tyr-Gly-Ser-Tyr-Ala-Tyr (SEQ ID NO:62), Ala-Arg-  
10 His-Asn-Tyr-Gly-Ser-Phe-Asp-Tyr (SEQ ID NO:64), Ala-Arg-  
His-Asn-Tyr-Gly-Ser-Phe-Tyr-Tyr (SEQ ID NO:66), Ala-Arg-  
20 His-Asn-Tyr-Gly-Ser-Phe-Ala-Ser (SEQ ID NO:68), Ala-Arg-  
His-Asn-Tyr-Gly-Ser-Phe-Ala-Thr (SEQ ID NO:70), Ala-Arg-  
His-Asn-Tyr-Gly-Ser-Phe-Ala-Asp (SEQ ID NO:72), Ala-Arg-  
15 His-Asn-Tyr-Gly-Ser-Phe-Ala-Glu (SEQ ID NO:74), Ala-Arg-  
25 His-Asn-Tyr-Gly-Ser-Phe-Ala-Met (SEQ ID NO:76), Ala-Arg-  
His-Asn-Tyr-Gly-Ser-Phe-Ala-Gly (SEQ ID NO:78) and Ala-  
Arg-His-Asn-Tyr-Gly-Ser-Phe-Ala-Ala (SEQ ID NO:80);  
the V<sub>L</sub> CDR1 Gln-Ala-Ser-Gln-Ser-Ile-Ser-Asn-Phe-Leu-His-  
30 Trp-Tyr (SEQ ID NO:82); the V<sub>L</sub> CDR2 Leu-Leu-Ile-Arg-Tyr-  
Ser-Ser-Gln-Ser-Ile-Ser (SEQ ID NO:84); and  
a V<sub>L</sub> CDR3 selected from the group consisting of Gln-Gln-  
35 Ser-Asn-Ser-Trp-Pro-His-Thr (SEQ ID NO:86), Gln-Gln-Ser-  
Thr-Ser-Trp-Pro-His-Thr (SEQ ID NO:88), Gln-Gln-Ser-Gly-  
25 Ser-Trp-Pro-Leu-Thr (SEQ ID NO:90) and Gln-Gln-Ser-Gly-  
Ser-Trp-Pro-Gln-Thr (SEQ ID NO:92).

40 The nucleotide sequences encoding the CDRs  
having single amino acid substitutions were V<sub>H</sub> CDR1 GGA  
ACT ACC TTC AGT AGC TAT GAC ATG TCT (SEQ ID NO:47), GGA  
45 30 TTC ACC TGG AGT AGC TAT GAC ATG TCT (SEQ ID NO:49), and  
GGA TTC ACC TTC CTG AGC TAT GAC ATG TCT (SEQ ID NO:51); V<sub>H</sub>  
CDR2 TGG GTC GCA AAA GTT AAA AGT GGT GGT GGT (SEQ ID  
NO:53), AGC ACC TAC TAT CCT GAC ACT GTG CAG GGC (SEQ ID  
50 NO:55), and AGC ACC TAC TAT TTA GAC ACT GTG GAG GGC (SEQ  
55

5 ID NO:57); V<sub>H</sub> CDR3 GCA AGA CAT AAC CAT GGC AGT TTT GCT TAC  
(SEQ ID NO:59), GCA AGA CAT AAC TAC GGC AGT TAT GCT TAC  
(SEQ ID NO:61), GCA AGA CAT AAC TAC GGC AGT TTT GAT TAC  
10 (SEQ ID NO:63), GCA AGA CAT AAC TAC GGC AGT TTT TAT TAC  
5 (SEQ ID NO:65), GCA AGA CAT AAC TAC GGC AGT TTT GCT TCT  
(SEQ ID NO:67), GCA AGA CAT AAC TAC GGC AGT TTT GCT ACT  
(SEQ ID NO:69), GCA AGA CAT AAC TAC GGC AGT TTT GCT GAT  
15 (SEQ ID NO:71), GCA AGA CAT AAC TAC GGC AGT TTT GCT GAG  
(SEQ ID NO:73), GCA AGA CAT AAC TAC GGC AGT TTT GCT ATG  
10 (SEQ ID NO:75), GCA AGA CAT AAC TAC GGC AGT TTT GCT GGG  
(SEQ ID NO:77), and GCA AGA CAT AAC TAC GGC AGT TTT GCT  
20 GCT (SEQ ID NO:79); V<sub>L</sub> CDR1 CAG GCC AGC CAA AGT ATT AGC  
AAC TTT CTA CAC TGG TAT (SEQ ID NO:81); V<sub>L</sub> CDR2 CTT CTC  
ATC CGT TAT TCT TCC CAG TCC ATC TCT (SEQ ID NO:83); and V<sub>L</sub>  
25 CDR3 CAA CAG AGT AAT AGC TGG CCT CAC ACG (SEQ ID NO:85),  
CAA CAG AGT ACT AGC TGG CCT CAC ACG (SEQ ID NO:87), CAA  
CAG AGT GGC AGC TGG CCT CTG ACG (SEQ ID NO:89) and CAA  
CAG AGT GGC AGC TGG CCT CAG ACG (SEQ ID NO:91).

30  
Enhanced LM609 grafted antibodies having CDRs  
20 with single amino acid substitutions and higher affinity  
binding than the parent LM609 grafted antibody can also  
35 be identified, where the corresponding amino acid  
mutations are combined to generate new modified LM609  
grafted antibodies. Identification is performed by  
25 screening for  $\alpha\beta$  binding activity. In some  
40 combinations, the LM609 grafted antibody will comprise at  
least one CDR having two or more amino acid  
substitutions. The invention provides an enhanced LM609  
grafted antibody containing at least one of the following  
45 30 CDRs containing multiple amino acid substitutions: a V<sub>H</sub>  
CDR3 selected from the group consisting of Ala-Arg-His-  
Asn-His-Gly-Ser-Phe-Ala-Ser (SEQ ID NO:94); Ala-Arg-His-  
Asn-His-Gly-Ser-Phe-Tyr-Ser (SEQ ID NO:96); Ala-Arg-His-  
50

Asn-Tyr-Gly-Ser-Phe-Tyr-Glu (SEQ ID NO:98); and Ala-Arg-His-Asn-Tyr-Gly-Ser-Phe-Tyr-Ser (SEQ ID NO:100).

The nucleotide sequences encoding the CDRS having multiple amino acid substitutions were V<sub>H</sub> CDR3 GCA  
5 AGA CAT AAC CAT GGC AGT TTT GCT TCT (SEQ ID NO:93), GCA  
AGA CAT AAC CAT GGC AGT TTT TAT TCT (SEQ ID NO:95), GCA  
15 AGA CAT AAC TAC GGC AGT TTT TAT GAG (SEQ ID NO:97), and  
GCA AGA CAT AAC TAC GGC AGT TTT TAT TCT (SEQ ID NO:99).

The invention also provides an enhanced LM609  
20 grafted antibody exhibiting selective binding affinity to  $\alpha_v\beta_3$ , wherein the enhanced LM609 grafted antibody contains  
at least one amino acid substitution in two or more CDRs  
25 of a LM609 grafted heavy chain variable region  
polypeptide or a LM609 grafted light chain variable  
15 region polypeptide.

An enhanced LM609 grafted antibody containing  
30 at least one amino acid substitution in two or more CDRs  
of a LM609 grafted heavy chain variable region  
polypeptide or a LM609 grafted light chain variable  
35 region polypeptide can include an LM609 grafted antibody  
containing the combination of CDRs selected from the  
group consisting of: the V<sub>L</sub> CDR1 SEQ ID NO:57 and the V<sub>H</sub>  
CDR3 SEQ ID NO:50; the V<sub>L</sub> CDR1 SEQ ID NO:57, the V<sub>H</sub> CDR2  
40 SEQ ID NO:44 and the V<sub>H</sub> CDR3 SEQ ID NO:50; the V<sub>L</sub> CDR1 SEQ  
25 ID NO:57, the V<sub>H</sub> CDR2 SEQ ID NO:44 and the V<sub>H</sub> CDR3 SEQ ID  
NO:52; the V<sub>L</sub> CDR1 SEQ ID NO:57, the V<sub>H</sub> CDR2 SEQ ID NO:44  
and the V<sub>H</sub> CDR3 SEQ ID NO:51; the V<sub>L</sub> CDR1 SEQ ID NO:57 and  
45 the V<sub>H</sub> CDR3 SEQ ID NO:52; the V<sub>L</sub> CDR3 SEQ ID NO:59, the V<sub>H</sub>  
CDR2 SEQ ID NO:44 and the V<sub>H</sub> CDR3 SEQ ID NO:50; the V<sub>L</sub>  
30 CDR3 SEQ ID NO:61 and V<sub>H</sub> CDR3 SEQ ID NO:50; and the V<sub>L</sub>  
CDR3 SEQ ID NO:61, the V<sub>H</sub> CDR2 SEQ ID NO:44 and V<sub>H</sub> CDR3  
50 SEQ ID NO:50.

5 In addition to enhanced LM609 grafted  
antibodies containing two or more CDRs having single  
amino acid substitutions, the invention also provides  
10 enhanced LM609 grafted antibodies wherein at least one of  
5 the CDRs has two or more amino acid substitutions.

15 Enhanced LM609 grafted antibodies having at  
least one CDR with two or more amino acid substitutions  
can include those containing the combination of CDRs  
selected from the group consisting of: the  $V_L$  CDR1 SEQ ID  
10 NO:57, the  $V_H$  CDR2 SEQ ID NO:44 and the  $V_H$  CDR3 SEQ ID  
20 NO:63; the  $V_L$  CDR3 SEQ ID NO:61, the  $V_H$  CDR2 SEQ ID NO:44  
and the  $V_H$  CDR3 SEQ ID NO:63; the  $V_L$  CDR3 SEQ ID NO:61,  
the  $V_H$  CDR2 SEQ ID NO:44 and the  $V_H$  CDR3 SEQ ID NO:64; the  
25  $V_L$  CDR3 SEQ ID NO:61 and the  $V_H$  CDR3 SEQ ID NO:63; the  $V_L$   
15 CDR3 SEQ ID NO:61 and the  $V_H$  CDR3 SEQ ID NO:65; and the  $V_L$   
CDR3 SEQ ID NO:61, the  $V_H$  CDR2 SEQ ID NO:44 and the  $V_H$   
CDR3 SEQ ID NO:66.

30 The invention additionally provides a high  
affinity LM609 grafted antibody exhibiting selective  
20 binding affinity to  $\alpha_v\beta_3$ . The high affinity LM609 grafted  
35 antibody contains at least one amino acid substitution in  
one or more CDRs of a LM609 grafted heavy chain variable  
region polypeptide or a LM609 grafted light chain  
variable region polypeptide, wherein the  $\alpha_v\beta_3$  binding  
40 25 affinity of the high affinity LM609 grafted antibody is  
enhanced.

45 High affinity antibodies can include those  
containing the combination of CDRs selected from the  
group consisting of: the  $V_L$  CDR1 SEQ ID NO:57 and the  $V_H$   
30 CDR3 SEQ ID NO:50; the  $V_L$  CDR1 SEQ ID NO:57, the  $V_H$  CDR2  
SEQ ID NO:44 and the  $V_H$  CDR3 SEQ ID NO:50; the  $V_L$  CDR1 SEQ  
50 ID NO:57, the  $V_H$  CDR2 SEQ ID NO:44 and the  $V_H$  CDR3 SEQ ID



5 NO:52; the V<sub>L</sub> CDR1 SEQ ID NO:57, the V<sub>H</sub> CDR2 SEQ ID NO:44  
and the V<sub>H</sub> CDR3 SEQ ID NO:51; the V<sub>L</sub> CDR1 SEQ ID NO:57 and  
the V<sub>H</sub> CDR3 SEQ ID NO:52; the V<sub>L</sub> CDR3 SEQ ID NO:59, the V<sub>H</sub>  
10 CDR2 SEQ ID NO:44 and the V<sub>H</sub> CDR3 SEQ ID NO:50; the V<sub>L</sub>  
5 CDR3 SEQ ID NO:61, the V<sub>H</sub> CDR2 SEQ ID NO:44 and the V<sub>H</sub>  
CDR3 SEQ ID NO:63; the V<sub>L</sub> CDR3 SEQ ID NO:61 and V<sub>H</sub> CDR3  
SEQ ID NO:50; the V<sub>L</sub> CDR3 SEQ ID NO:61, the V<sub>H</sub> CDR2 SEQ ID  
15 NO:44 and V<sub>H</sub> CDR3 SEQ ID NO:50; the V<sub>L</sub> CDR1 SEQ ID NO:57,  
the V<sub>H</sub> CDR2 SEQ ID NO:44 and the V<sub>H</sub> CDR3 SEQ ID NO:63; the  
20 V<sub>L</sub> CDR3 SEQ ID NO:61, the V<sub>H</sub> CDR2 SEQ ID NO:44 and the V<sub>H</sub>  
CDR3 SEQ ID NO:64; the V<sub>L</sub> CDR3 SEQ ID NO:61 and the V<sub>H</sub>  
CDR3 SEQ ID NO:63; the V<sub>L</sub> CDR3 SEQ ID NO:61 and the V<sub>H</sub>  
CDR3 SEQ ID NO:65; and the V<sub>L</sub> CDR3 SEQ ID NO:61, the V<sub>H</sub>  
CDR2 SEQ ID NO:44 and the V<sub>H</sub> CDR3 SEQ ID NO:66.

25 15 As described above, enhanced LM609 antibodies  
can be further modified by introducing additional  
mutations in one or more CDRs or framework residues. As  
30 disclosed herein, the enhanced LM609 grafted antibody  
clone 6H6 (Table 10) was further modified by introducing  
20 mutations into one or more CDRs (see Example VIII).  
These 6H6 variants were found to have high affinity  
35 binding to  $\alpha_v\beta_3$  and were resistant to proteolysis.

The invention further provides enhanced LM609  
grafted antibodies comprising the V<sub>H</sub> CDR2 Lys-Val-Ser-Ser-  
40 25 Gly-Gly-Gly-Ser-Thr-Tyr-Tyr-Pro-Asp-Thr-Val-Gln-Gly (SEQ  
ID NO:104); the V<sub>H</sub> CDR3 His-Leu-His-Gly-Ser-Phe-Ala-Ser  
(SEQ ID NO:106); or the V<sub>L</sub> CDR1 Gln-Ala-Ser-Gln-Ser-Ile-  
Ser-Asn-Phe-Leu-His (SEQ ID NO:110). The invention also  
45 provides a nucleic acid molecule comprising a nucleotide  
30 sequence selected from the group consisting of SEQ ID  
NO:103, SEQ ID NO:105, and SEQ ID NO:109.

5

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The invention also provides an enhanced LM609 grafted antibody comprising the V<sub>H</sub> CDR1 Gly-Phe-Thr-Phe-Ser-Ser-Tyr-Asp-Met-Ser (SEQ ID NO:34); the V<sub>H</sub> CDR2 Lys-Val-Ser-Ser-Gly-Gly-Gly-Ser-Thr-Tyr-Tyr-Leu-Asp-Thr-Val-Gln-Gly (SEQ ID NO:102); the V<sub>H</sub> CDR3 His-Leu-His-Gly-Ser-Phe-Ala-Ser (SEQ ID NO:106); the V<sub>L</sub> CDR1 Gln-Ala-Ser-Gln-Ser-Ile-Ser-Asn-His-Leu-His (SEQ ID NO:108); the V<sub>L</sub> CDR2 Tyr-Arg-Ser-Gln-Ser-Ile-Ser (SEQ ID NO:112); and the V<sub>L</sub> CDR3 Gln-Gln-Ser-Gly-Ser-Trp-Pro-Leu-Thr (SEQ ID NO:90).

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10 The invention also provides a nucleic acid molecule comprising the nucleotide sequence referenced as SEQ ID NO:33 encoding a V<sub>H</sub> CDR1; the nucleotide sequence referenced as SEQ ID NO:101 encoding a V<sub>H</sub> CDR2; the nucleotide sequence referenced as SEQ ID NO:105 encoding a V<sub>H</sub> CDR3; the nucleotide sequence referenced as SEQ ID NO:107 encoding a V<sub>L</sub> CDR1; the nucleotide sequence referenced as SEQ ID NO:111 encoding a V<sub>L</sub> CDR2; and the nucleotide sequence referenced as SEQ ID NO:89 encoding a V<sub>L</sub> CDR3.

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20 The invention additionally provides an enhanced LM609 grafted antibody comprising the V<sub>H</sub> CDR1 Gly-Phe-Thr-Phe-Ser-Ser-Tyr-Asp-Met-Ser (SEQ ID NO:34); the V<sub>H</sub> CDR2 Lys-Val-Ser-Ser-Gly-Gly-Gly-Ser-Thr-Tyr-Tyr-Leu-Asp-Thr-Val-Gln-Gly (SEQ ID NO:102); the V<sub>H</sub> CDR3 His-Leu-His-Gly-Ser-Phe-Ala-Ser (SEQ ID NO:106); the V<sub>L</sub> CDR1 Gln-Ala-Ser-Gln-Ser-Ile-Ser-Asn-Phe-Leu-His (SEQ ID NO:110); the V<sub>L</sub> CDR2 Tyr-Arg-Ser-Gln-Ser-Ile-Ser (SEQ ID NO:112); and the V<sub>L</sub> CDR3 Gln-Gln-Ser-Gly-Ser-Trp-Pro-Leu-Thr (SEQ ID NO:90).

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30 The invention additionally provides a nucleic acid molecule comprising the nucleotide sequence referenced as SEQ ID NO:33 encoding a V<sub>H</sub> CDR1; the

5 nucleotide sequence referenced as SEQ ID NO:101 encoding  
a V<sub>H</sub> CDR2; the nucleotide sequence referenced as SEQ ID  
10 NO:105 encoding a V<sub>H</sub> CDR3; the nucleotide sequence  
referenced as SEQ ID NO:109 encoding a V<sub>L</sub> CDR1; the  
5 nucleotide sequence referenced as SEQ ID NO:111 encoding  
a V<sub>L</sub> CDR2; and the nucleotide sequence referenced as SEQ  
ID NO:89 encoding a V<sub>L</sub> CDR3.

15 The invention further provides an enhanced  
LM609 grafted antibody comprising the V<sub>H</sub> CDR1 Gly-Phe-Thr-  
10 Phe-Ser-Ser-Tyr-Asp-Met-Ser (SEQ ID NO:34); the V<sub>H</sub> CDR2  
Lys-Val-Ser-Ser-Gly-Gly-Gly-Ser-Thr-Tyr-Tyr-Pro-Asp-Thr-  
20 Val-Gln-Gly (SEQ ID NO:104); the V<sub>H</sub> CDR3 His-Leu-His-Gly-  
Ser-Phe-Ala-Ser (SEQ ID NO:106); the V<sub>L</sub> CDR1 Gln-Ala-Ser-  
Gln-Ser-Ile-Ser-Asn-Phe-Leu-His (SEQ ID NO:110); the V<sub>L</sub>  
25 CDR2 Tyr-Arg-Ser-Gln-Ser-Ile-Ser (SEQ ID NO:112); and the  
V<sub>L</sub> CDR3 Gln-Gln-Ser-Gly-Ser-Trp-Pro-Leu-Thr (SEQ ID  
NO:90).

30 The invention further provides a nucleic acid  
molecule comprising the nucleotide sequence referenced as  
20 SEQ ID NO:33 encoding a V<sub>H</sub> CDR1; the nucleotide sequence  
referenced as SEQ ID NO:103 encoding a V<sub>H</sub> CDR2; the  
35 nucleotide sequence referenced as SEQ ID NO:105 encoding  
a V<sub>H</sub> CDR3; the nucleotide sequence referenced as SEQ ID  
NO:109 encoding a V<sub>L</sub> CDR1; the nucleotide sequence  
40 25 referenced as SEQ ID NO:111 encoding a V<sub>L</sub> CDR2; and the  
nucleotide sequence referenced as SEQ ID NO:89 encoding a  
V<sub>L</sub> CDR3.

45 The invention additionally provides a nucleic  
acid encoding an enhanced LM609 grafted antibody  
30 exhibiting selective binding affinity to  $\alpha_v\beta_3$ . The  
enhanced LM609 grafted antibody encoded by the nucleic  
50 acid contains at least one amino acid substitution in one

5 or more CDRs of a LM609 grafted heavy chain variable  
region polypeptide or a LM609 grafted light chain  
variable region polypeptide, wherein the  $\alpha_v\beta_3$  binding  
10 affinity of the enhanced LM609 grafted antibody is  
5 maintained or enhanced.

15 The invention further provides a nucleic acid  
encoding a high affinity LM609 grafted antibody  
exhibiting selective binding affinity to  $\alpha_v\beta_3$ . The high  
affinity LM609 grafted antibody encoded by the nucleic  
20 acid contains at least one amino acid substitution in one  
or more CDRs of a LM609 grafted heavy chain variable  
region polypeptide or a LM609 grafted light chain  
variable region polypeptide, wherein the  $\alpha_v\beta_3$  binding  
25 affinity of the high affinity LM609 grafted antibody is  
15 enhanced.

30 The invention provides a nucleic acid encoding  
a heavy chain polypeptide for monoclonal antibody LM609  
or functional fragment thereof. Also provided is a  
nucleic acid encoding a light chain polypeptide for  
20 monoclonal antibody LM609 or a functional fragment  
thereof. The nucleic acids consist of substantially the  
35 same heavy or light chain variable region nucleotide  
sequences as that shown in Figure 2A and 2B (SEQ ID NOS:5  
and 7, respectively) or a fragment thereof.

40 25 As described previously, monoclonal antibody  
LM609 has been shown in the art to have binding activity  
to the integrin  $\alpha_v\beta_3$ . Although specificity can in  
45 principle be generated towards essentially any target,  
LM609 is an integrin inhibitory antibody that exhibits  
30 substantial specificity and inhibitory activity to a  
single member within an integrin family. In this case,  
50 LM609 exhibits substantial specificity and inhibitory

activity to the  $\alpha_v\beta_3$  integrin within the  $\beta_3$  family. The amino acid or nucleotide sequence of monoclonal antibody LM609 has never been previously isolated and characterized.

The isolation and characterization of LM609 encoding nucleic acids was performed by techniques known to those skilled in the art and which are described further below in the Examples. Briefly, cDNA from hybridoma LM609 was generated and used as the source for which to isolate LM609 encoding nucleic acids. Isolation was performed by first determining the N-terminal amino acid sequence for each of the heavy and light chain polypeptides and then amplifying by PCR the antibody encoding sequences from the cDNA. The 5' primers were reverse translated to correspond to the newly determined N-terminal amino acid sequences whereas the 3' primers corresponded to sequences substantially similar to antibody constant region sequences. Amplification and cloning of the products resulted in the isolation of the nucleic acids encoding heavy and light chains of LM609.

The nucleotide sequences of the LM609 heavy and light chain variable region sequences are shown in Figure 2A and 2B, respectively. These sequences correspond substantially to those that encode the variable region heavy and light chain polypeptides of LM609. As with the Vitaxin nucleic acids, these LM609 nucleic acids are intended to include both sense and anti-sense strands of the LM609 encoding sequences. Single- and double-stranded nucleic acids are also include as well as non-coding portions of the nucleic acid such as introns, 5'- and 3'-untranslated regions and regulatory sequences of the gene for example.

5 As shown in Figure 2A, the LM609 heavy chain  
variable region polypeptide is encoded by a nucleic acid  
of about 351 nucleotides in length which begins at the  
10 amino terminal Glu1 residue of the variable region  
5 through to Ala 117. The murine LM609 antibody heavy  
chain has an IgG2a constant region. Shown in Figure 2B  
is the LM609 light chain variable region polypeptide  
15 which is encoded by a nucleic acid of about 321  
nucleotides in length which begins at the amino terminal  
10 Asp1 residue of the variable region through to Lys 107.  
In the functional antibody, LM609 has a kappa light chain  
20 constant region.

As with the Vitaxin nucleic acids, minor  
25 modifications of these LM609 nucleotide sequences are  
15 intended to be included as heavy and light chain LM609  
encoding nucleic acids. Such minor modifications are  
included within the nucleic acids encoding LM609 heavy  
30 and light chain polypeptides so long as the nucleic acids  
or encoded polypeptides retain some or all of their  
20 function as described.

35 Thus, the invention also provides a nucleic  
acid encoding a LM609 heavy chain or functional fragment  
wherein the nucleic acid encodes substantially the same  
variable region amino acid sequence of monoclonal  
40 25 antibody LM609 as that shown in Figure 2A (SEQ ID NO:6)  
or a fragment thereof. Similarly, the invention also  
provides a nucleic acid encoding a LM609 light chain or  
functional fragment wherein the nucleic acid encodes  
45 substantially the same variable region amino acid  
30 sequence of monoclonal antibody LM609 as that shown in  
Figure 2B (SEQ ID NO:8) or a fragment thereof.

5 The invention further provides fragments of  
LM609 heavy and light chain encoding nucleic acids  
wherein such fragments consist substantially of the same  
10 nucleotide or amino acid sequence as the variable region  
5 of LM609 heavy or light chain polypeptides. The variable  
region of the LM609 heavy chain polypeptide consists  
essentially of nucleotides 1-351 and of amino acid  
15 residues Glu1 to Ala117 of Figure 2A (SEQ ID NOS:5 and 6,  
respectively). The variable region of the LM609 light  
20 chain polypeptide consists essentially of nucleotides  
1-321 and of amino acid residues Asp1 to Lys107 of Figure  
2B (SEQ ID NOS:7 and 8, respectively). The termini of  
such variable region encoding nucleic acids is not  
critical so long as the intended purpose and function  
25 remains the same. Such intended purposes and functions  
include, for example, use for the production of  
recombinant polypeptides or as hybridization probes for  
heavy and light chain variable region sequences.

30 Fragments additional to the variable region  
20 nucleic acid fragments are provided as well. Such  
fragments include, for example, nucleic acids consisting  
35 substantially of the same nucleotide sequence as a CDR of  
a LM609 heavy or light chain polypeptide. Sequences  
corresponding to the LM609 CDRs include, for example,  
25 those regions within the variable region which are  
40 defined by Kabat et al., *supra*, and/or those regions  
within the variable regions which are defined by Chothia  
et al., *supra*, as well as those regions defined by  
MacCallum et al., *supra*. The LM609 CDR fragments for  
45 30 each of the above definitions correspond to the  
nucleotides set forth below in Table 4. The nucleotide  
sequence numbering is taken from the primary sequence  
shown in Figures 2A and 2B (SEQ ID NOS:5 and 7) and  
50

conforms to the definitions previously set forth in Table 1.

**Table 4: LM609 CDR Nucleotide Residues**

	<u>Kabat</u>	<u>Chothia</u>	<u>MacCallum</u>
V <sub>H</sub> CDR1	91-105	76-96	88-105
V <sub>H</sub> CDR2	148-198	157-168	139-177
V <sub>H</sub> CDR3	295-318	298-315	288-315
V <sub>L</sub> CDR1	70-102	76-96	88-108
V <sub>L</sub> CDR2	148-168	148-156	136-165
V <sub>L</sub> CDR3	265-291	271-288	265-288

Similarly, the LM609 CDR fragments for each of the above definitions correspond to the amino acid residues set forth below in Table 5. The amino acid residue numbering is taken from the primary sequence shown in Figures 2A and 2B (SEQ ID NOS:6 and 8) and conforms to the definitions set forth in Table 1.

**Table 5: LM609 CDR Amino Acid Residues**

	<u>Kabat</u>	<u>Chothia</u>	<u>MacCallum</u>
V <sub>H</sub> CDR1	Ser31-Ser35	Gly26-Tyr32	Ser30-Ser35
V <sub>H</sub> CDR2	Lys50-Gly66	Ser53-Gly56	Trp47-Tyr59
V <sub>H</sub> CDR3	His99-Tyr106	Asn100-Ala105	Ala97-Ala105
V <sub>L</sub> CDR1	Gln24-His34	Ser26-His32	Ser30-Tyr36
V <sub>L</sub> CDR2	Tyr50-Ser56	Tyr50-Ser52	Leu46-Ile55
V <sub>L</sub> CDR3	Gln89-Thr97	Ser91-His96	Gln89-His96

Nucleic acids encoding LM609 heavy and light chain polypeptides and fragments thereof are useful for a variety of diagnostic and therapeutic purposes. For example, the LM609 nucleic acids can be used to produce recombinant LM609 antibodies and functional fragments thereof having binding specificity and inhibitory



activity against the integrin  $\alpha_v\beta_3$ . The antibody and functional fragments thereof can be used to determine the presence or absence of  $\alpha_v\beta_3$  in a sample to diagnose the susceptibility or occurrence of an  $\alpha_v\beta_3$ -mediated disease.

Alternatively, the recombinant LM609 antibodies and functional fragments thereof can be used for the therapeutic treatment of  $\alpha_v\beta_3$ -mediated diseases or pathological state. As with Vitaxin, recombinant LM609 and functional fragments thereof can be used to inhibit the binding activity or other functional activities of  $\alpha_v\beta_3$  that are necessary for progression of the  $\alpha_v\beta_3$ -mediated disease or pathological state.

The LM609 nucleic acids of the invention can also be used to model functional equivalents of the encoded heavy and light chain polypeptides. Such functional equivalents can include, for example, synthetic analogues or mimics of the encoded polypeptides or functional fragments thereof. A specific example would include peptide mimetics of the LM609 CDRs that retain some or substantially the same binding or inhibitory activity of LM609. Additionally, the LM609 encoding nucleic acids can be used to engineer and produce nucleic acids which encode modified forms or derivatives of the antibody LM609, its heavy and light chain polypeptides and functional fragments thereof. As described previously, such modified forms or derivatives include, for example, non-mouse antibodies, their corresponding heavy and light chain polypeptides and functional fragments thereof which exhibit substantially the same binding and inhibitory activity as LM609.

The invention also provides a method of treating an  $\alpha_v\beta_3$ -mediated disease. The method consists of administering an effective amount of Vitaxin, a LM609

5 grafted antibody, an enhanced antibody thereof, or a  
functional fragment thereof under conditions which allow  
binding to  $\alpha_v\beta_3$ . Also provided is a method of inhibiting  
10 a function of  $\alpha_v\beta_3$ . The method consists of contacting  $\alpha_v\beta_3$   
5 with Vitaxin, a LM609 grafted antibody or a functional  
fragment thereof under conditions which allow binding to  
 $\alpha_v\beta_3$ .

15 As described previously, Vitaxin and LM609  
grafted antibodies are monoclonal antibodies which  
10 exhibit essentially all of the binding characteristics as  
does its parental CDR-donor antibody LM609. These  
characteristics include, for example, significant binding  
specificity and affinity for the integrin  $\alpha_v\beta_3$ . The  
25 Examples below demonstrate these binding properties and  
15 further show that the binding of such antibodies to  $\alpha_v\beta_3$   
inhibits  $\alpha_v\beta_3$  ligand binding and function. Thus, Vitaxin  
and LM609 grafted antibodies are useful for a large  
variety of diagnostic and therapeutic purposes directed  
30 to the inhibition of  $\alpha_v\beta_3$  function.

20 The integrin  $\alpha_v\beta_3$  functions in numerous cell  
adhesion and migration associated events. As such, the  
35 dysfunction or dysregulation of this integrin, its  
function, or of cells expressing this integrin, is  
associated with a large number of diseases and  
40 25 pathological conditions. The inhibition  $\alpha_v\beta_3$  binding or  
function can therefore be used to treat or reduce the  
severity of such  $\alpha_v\beta_3$ -mediated pathological conditions.  
Described below are examples of several pathological  
45 conditions mediated by  $\alpha_v\beta_3$ , since the inhibition of at  
30 least this integrin reduces the severity of the  
condition. These examples are intended to be  
representative and as such are not inclusive of all  
50  $\alpha_v\beta_3$ -mediated diseases. For example, there are numerous

5 pathological conditions additional to those discussed  
below which exhibit the dysregulation of  $\alpha_v\beta_3$  binding,  
function or the dysregulation of cells expressing this  
10 integrin and in which the pathological condition can be  
5 reduced, or will be found to be reduced, by inhibiting  
the binding  $\alpha_v\beta_3$ . Such pathological conditions which  
exhibit this criteria, are intended to be included within  
15 the definition of the term as used herein.

Angiogenesis, or neovascularization, is the  
10 process where new blood vessels form from pre-existing  
20 vessels within a tissue. As described further below,  
this process is mediated by endothelial cells expressing  
 $\alpha_v\beta_3$  and inhibition of at least this integrin, inhibits  
25 new vessel growth. There are a variety of pathological  
15 conditions that require new blood vessel formation or  
tissue neovascularization and inhibition of this process  
inhibits the pathological condition. As such,  
30 pathological conditions that require neovascularization  
for growth or maintenance are considered to be  
20  $\alpha_v\beta_3$ -mediated diseases. The extent of treatment, or  
reduction in severity, of these diseases will therefore  
35 depend on the extent of inhibition of neovascularization.  
These  $\alpha_v\beta_3$ -mediated diseases include, for example,  
inflammatory disorders such as immune and non-immune  
25 inflammation, chronic articular rheumatism, psoriasis,  
40 disorders associated with inappropriate or inopportune  
invasion of vessels such as diabetic retinopathy,  
neovascular glaucoma and capillary proliferation in  
atherosclerotic plaques as well as cancer disorders.  
45 30 Such cancer disorders can include, for example, solid  
tumors, tumor metastasis, angiofibromas, retrolental,  
fibroplasia, hemangiomas, Kaposi's sarcoma and other  
cancers which require neovascularization to support tumor  
50 growth. Additional diseases which are considered

5 angiogenic include psoriasis and rheumatoid arthritis as  
well as retinal diseases such as macular degeneration.  
Diseases other than those requiring new blood vessels  
10 which are  $\alpha_v\beta_3$ -mediated diseases include, for example,  
5 restenosis and osteoporosis.

15 Treatment of the  $\alpha_v\beta_3$ -mediated diseases can be  
performed by administering an effective amount of  
Vitaxin, a LM609 grafted antibody, an enhanced antibody  
thereof, or a functional fragment thereof so as to bind  
20 to  $\alpha_v\beta_3$  and inhibit its function. Administration can be  
performed using a variety of methods known in the art.  
The choice of method will depend on the specific  $\alpha_v\beta_3$ -  
mediated disease and can include, for example, the *in*  
25 *vivo*, *in situ* and *ex vivo* administration of Vitaxin, a  
15 LM609 grafted antibody or functional fragment thereof, to  
cells, tissues, organs, and organisms. Moreover, such  
antibodies or functional fragments can be administered to  
an individual exhibiting or at risk of exhibiting an  $\alpha_v\beta_3$ -  
30 mediated disease. Definite clinical diagnosis of an  $\alpha_v\beta_3$ -  
20 mediated disease warrants the administration of Vitaxin,  
a LM609 grafted antibody or a functional fragment  
thereof. Prophylactic applications are warranted in  
35 diseases where the  $\alpha_v\beta_3$ -mediated disease mechanisms  
precede the onset of overt clinical disease. Thus,  
25 individuals with familial history of disease and  
40 predicted to be at risk by reliable prognostic indicators  
can be treated prophylactically to interdict  $\alpha_v\beta_3$ -mediated  
mechanisms prior to their onset.

45 Vitaxin, a LM609 grafted antibody, an enhanced  
30 antibody thereof, or functional fragments thereof can be  
administered in a variety of formulations and  
pharmaceutically acceptable media for the effective  
50 treatment or reduction in the severity of an  $\alpha_v\beta_3$ -mediated

5 disease. Such formulations and pharmaceutically  
acceptable medias are well known to those skilled in the  
art. Additionally, Vitaxin, a LM609 grafted antibody or  
10 functional fragments thereof can be administered with  
5 other compositions which can enhance or supplement the  
treatment or reduction in severity of an  $\alpha_v\beta_3$ -mediated  
disease. For example, the coadministration of Vitaxin or  
15 a LM609 grafted antibody to inhibit tumor-induced  
neovascularization and a chemotherapeutic drug to  
10 directly inhibit tumor growth is one specific case where  
the administration of other compositions can enhance or  
20 supplement the treatment of an  $\alpha_v\beta_3$ -mediated disease.

Vitaxin, a LM609 grafted antibody or functional  
25 fragments are administered by conventional methods, in  
15 dosages which are sufficient to cause the inhibition of  
 $\alpha_v\beta_3$  integrin binding at the sight of the pathology.  
Inhibition can be measured by a variety of methods known  
30 in the art such as *in situ* immunohistochemistry for the  
prevalence of  $\alpha_v\beta_3$  containing cells at the site of the  
20 pathology as well as include, for example, the observed  
reduction in the severity of the symptoms of the  
35  $\alpha_v\beta_3$ -mediated disease.

*In vivo* modes of administration can include  
intraperitoneal, intravenous and subcutaneous  
40 administration of Vitaxin, a LM609 grafted antibody or a  
25 functional fragment thereof. Dosages for antibody  
therapeutics are known or can be routinely determined by  
those skilled in the art. For example, such dosages are  
45 typically administered so as to achieve a plasma  
30 concentration from about 0.01  $\mu\text{g/ml}$  to about 100  $\mu\text{g/ml}$ ,  
preferably about 1-5  $\mu\text{g/ml}$  and more preferably about 5  
50  $\mu\text{g/ml}$ . In terms of amount per body weight, these dosages  
typically correspond to about 0.1-300 mg/kg, preferably

5 about 0.2-200 mg/kg and more preferably about 0.5-20  
mg/kg. Depending on the need, dosages can be  
administered once or multiple times over the course of  
10 the treatment. Generally, the dosage will vary with the  
5 age, condition, sex and extent of the  $\alpha_v\beta_3$ -mediated  
pathology of the subject and should not be so high as to  
cause adverse side effects. Moreover, dosages can also  
15 be modulated by the physician during the course of the  
treatment to either enhance the treatment or reduce the  
10 potential development of side effects. Such procedures  
are known and routinely performed by those skilled in the  
20 art.

The specificity and inhibitory activity of  
25 Vitaxin, LM609 grafted antibodies, an enhanced antibody  
15 thereof and functional fragments thereof allow for the  
therapeutic treatment of numerous  $\alpha_v\beta_3$ -mediated diseases.  
Such diseases include, for example, pathological  
30 conditions requiring neovascularization such as tumor  
growth, and psoriasis as well as those directly mediated  
20 by  $\alpha_v\beta_3$ , such as restenosis and osteoporosis. Thus, the  
invention provides methods as well as Vitaxin and LM609  
35 grafted antibody containing compositions for the  
treatment of such diseases.

It is understood that modifications which do  
40 25 not substantially affect the activity of the various  
embodiments of this invention are also included within  
the definition of the invention provided herein.  
Accordingly, the following examples are intended to  
45 illustrate but not limit the present invention.

## EXAMPLE I

Isolation and Characterization of LM609Encoding Nucleic Acids

This Example shows the cloning and sequence determination of LM609 encoding nucleic acids.

LM609 is directed against the human vitronectin receptor, integrin  $\alpha_v\beta_3$ .  $\alpha_v\beta_3$  is highly upregulated in melanoma, glioblastoma, and mammary carcinoma and plays a role in the proliferation of M21 melanoma cells both in vitro and in vivo.  $\alpha_v\beta_3$  also plays a role in angiogenesis, restenosis and the formation of granulation tissue in cutaneous wounds. LM609 has been shown to inhibit the adhesion of M21 cells to vitronectin as well as prevent proliferation of M21 cells in vitro. Thus, grafting of LM609 could result in a clinically valuable therapeutic agent.

cDNA Synthesis of LM609 Variable Regions: For cDNA synthesis, total RNA was prepared from  $10^6$  LM609 hybridoma cells using a modification of the method described by Chomczynski and Sacchi (Chomczynski and Sacchi, Analyt. Biochem. 162:156 (1987)). LM609 variable (V) region genes were cloned by reverse transcription-polymerase chain reaction (RT-PCR) and cDNA was synthesized using BRL Superscript kit. Briefly, 5 mg of total cellular RNA, 650 ng oligo dT and  $H_2O$  were brought to a total volume of 55  $\mu$ l. The sample was heated to 70°C for 10 min and chilled on ice. Reaction buffer was added and the mixture brought to 10 mM DTT and 1 mM dNTPs and heated at 37°C for 2 minutes. 5  $\mu$ l (1000 units) reverse transcriptase was added and incubated at 37°C for 1 hour and then chilled on ice.

5 All oligonucleotides were synthesized by  
β-cyanoethyl phosphoramidite chemistry on an ABI 394 DNA  
synthesizer. Oligonucleotides used for PCR amplification  
10 and routine site-directed mutagenesis were purified using  
5 oligonucleotide purification cartridges (Applied  
Biosystems, Foster City, CA). Forward PCR primers were  
designed from N-terminal protein sequence data generated  
15 from purified LM609 antibody. The forward PCR primers  
contained sequences coding for the first six amino acids  
20 in each antibody variable chain (protein sequenced at San  
Diego State University). The sequence of the light chain  
forward PCR primer (997) was 5'-GCC CAA CCA GCC ATG GCC  
GAT ATT GTG CTA ACT CAG-3' (SEQ ID NO:19) whereas the  
light chain reverse PCR primer (734) was 5'-AC AGT TGG  
25 TGC AGC ATC AGC-3' (SEQ ID NO:20) used. This reverse  
primer corresponds to mouse light chain kappa amino acid  
residues 109-115. The sequence of the heavy chain  
forward PCR primer (998) was 5'-ACC CCT GTG GCA AAA GCC  
GAA GTG CAG CTG GTG GAG-3' (SEQ ID NO:21). Heavy chain  
30 reverse PCR primer 733: 5'-GA TGG GGG TGT CGT TTT GGC-3'  
SEQ ID NO:22). The PCR primers also contain regions of  
homology with specific sequences within the  
35 immunoexpression vector.

V<sub>L</sub> and V<sub>H</sub> chains were amplified in two separate  
25 50 ml reaction mixtures containing 2 ml of the cDNA-RNA  
heteroduplex, 66.6 mM Tris-HCl pH 8.8, 1.5 mM MgCl<sub>2</sub>, 0.2  
40 mM of each four dNTPs, 10 mM 2-mercaptoethanol, 0.25  
units Taq polymerase (Boehringer-Mannheim, Indianapolis,  
IN) and 50 pmoles each of primers 997 and 734 and 998 and  
45 733, respectively. The mixtures were overlaid with  
mineral oil and cycled for two rounds of PCR with each  
cycle consisting of 30 seconds at 94°C (denature), 30  
seconds at 50°C (anneal), and 30 seconds at 72°C  
50



(synthesis). This reaction was immediately followed by 30 cycles of PCR consisting of 30 seconds at 94°C (denature), 30 seconds at 55°C (anneal), and 30 seconds at 72°C (synthesis) followed by a final synthesis reaction for 5 minutes at 72°C. The reaction products were pooled, extracted with CHCl<sub>3</sub>, and ethanol precipitated.

Amplified products were resuspended in 20 ml TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) and electrophoresed on a 5% polyacrylamide gel. Bands migrating at expected molecular weights of V<sub>H</sub> and V<sub>L</sub> were excised, chemically eluted from the gel slice, extracted with organic solvents and ethanol precipitated.

Cloning of amplified V<sub>H</sub> and V<sub>L</sub> genes into M13 phage immunoexpression vector: The amplified V region gene products were sequentially cloned into the phage immunoexpression vector by hybridization mutagenesis (Near, R. Biotechniques 12:88 (1992); Yelton et al., J. Immunol. 155:1994-2003 (1995)). Introduction of the amplified V<sub>L</sub> and V<sub>H</sub> sequences by hybridization mutagenesis positions the antibody sequences in frame with the regulatory elements contained in the M13 vector required for efficient Fab expression. One advantage of this technique is that no restriction endonuclease sites need to be incorporated into the V<sub>L</sub> or V<sub>H</sub> gene sequences for cloning as is done with conventional DNA ligation methods.

To perform the cloning, 400 ng each of the double-stranded amplified products were first phosphorylated with polynucleotide kinase. 100 ng of the phosphorylated LM609 V<sub>L</sub> product was mixed with 250 ng of uridinylated BS11 phage immunoexpression vector, denatured by heating to 90°C and annealed by gradual

5 cooling to room temperature. BS11 is an M13  
immunoexpression vector derived from M13 IX and encodes  
CH<sub>1</sub> of murine IgG1 and murine kappa light chain constant  
10 domain (Huse, W.D. In: Antibody Engineering: A Practical  
5 Guide, C.A.K. Borrebaeck, ed. W.H. Freeman and Co.,  
Publishers, New York, pp. 103-120 (1991)). Nucleotide  
sequences included in the PCR amplification primers  
15 anneal to complementary sequences present in the single-  
stranded BS11 vector. The annealed mixture was fully  
20 converted to a double-stranded molecule with T4 DNA  
polymerase plus dNTPs and ligated with T4 ligase. 1 ml  
of the mutagenesis reaction was electroporated into *E.*  
25 *coli* strain DH10B, titered onto a lawn of XL-1 *E. coli*  
and incubated until plaques formed. Plaque lift assays  
were performed as described using goat anti-murine kappa  
30 chain antibody conjugated to alkaline phosphatase (Yelton  
et al, *supra*; Huse, W.D., *supra*). Fifteen murine light  
chain positive M13 phage clones were isolated, pooled and  
used to prepare uridynylated vector to serve as template  
20 for hybridization mutagenesis with the PCR amplified  
LM609 V<sub>H</sub> product.

35 Clones expressing functional murine LM609 Fab  
were identified by binding to purified a<sub>2</sub>b<sub>2</sub> by ELISA.  
Briefly, Immulon II ELISA plates were coated overnight  
25 with 1 mg/ml (100 ng/well) a<sub>2</sub>b<sub>2</sub> and nonspecific sites  
40 blocked for two hours at 27°C. Soluble Fabs were prepared  
by isolating periplasmic fractions of cultures of *E. coli*  
strain MK30-3 (Boehringer Mannheim Co.) infected with the  
Fab expressing M13 phage clones. Periplasm fractions  
45 30 were mixed with binding buffer 100 mM NaCl, 50 mM Tris pH  
7.4, 2mM CaCl<sub>2</sub>, 1 mM MgCl<sub>2</sub>, 1 mM MnCl<sub>2</sub>, 0.02% NaN<sub>3</sub>, 1 mg/ml  
BSA and incubated with immobilized a<sub>2</sub>b<sub>2</sub> for two hours at  
50 27°C. Plates were washed with binding buffer and bound

5 Fab detected with goat anti-murine kappa chain antibody  
conjugated to alkaline phosphatase. Four a<sub>1</sub>b<sub>1</sub> reactive  
clones were identified: muLM609M13 12, 29, 31 and 69.  
10 MuLM609M13 12 and 29 gave the strongest signals in the  
5 ELISA assay. DNA sequence analysis showed that clones  
muLM609M13 12, 31 and 69 all had identical light chain  
sequence and confirmed the previously determined  
15 N-terminal amino acid sequence of purified LM609 light  
chain polypeptide. All four clones had identical V<sub>H</sub> DNA  
20 sequence and also confirmed the previously determined  
N-terminal amino acid sequence of purified LM609 heavy  
chain polypeptide.

To further characterize the binding activity of  
each clone, soluble Fab fractions were prepared from 50  
25 ml cultures of *E. coli* strain MK30-3 infected with clones  
12 and 29 and evaluated for binding to a<sub>1</sub>b<sub>1</sub> in a  
competitive ELISA with LM609 IgG. The results of this  
30 ELISA are shown in Figure 3. Clone muLM609M13 12 was  
found to inhibit LM609 IgG binding (at LM609 IgG  
20 concentrations of 1 ng/ml and 5 ng/ml) to a<sub>1</sub>b<sub>1</sub> in a  
concentration dependent manner at periplasm titers  
ranging from neat to 1:80. Clone muLM609M13 12 was  
35 plaque purified and both the V region heavy and light  
chain DNA sequences again determined. Complete DNA  
25 sequence of the final clone, muLM609M13 12-5, is shown in  
40 Figures 2A and 2B.

#### EXAMPLE II

##### Construction of Vitaxin: A CDR Grafted

##### LM609 Functional Fragment

30 One goal of grafting antibodies is to preserve  
antibody specificity and affinity when substituting  
50

5 non-human CDRs into a human antibody framework. Another  
goal is to minimize the introduction of foreign amino  
acid sequences so as to reduce the possible antigenicity  
10 with a human host. This Example describes procedures for  
5 accomplishing both of these goals by producing libraries  
of grafted antibodies which represent all  
possible members which exhibit the highest affinities for  
15 the desired antigen.

The above library was constructed in *E. coli*  
10 wherein the possible CDR and framework changes were  
incorporated using codon-based mutagenesis (Kristensson  
et al., In: Vaccines 95. Cold Spring Harbor Laboratory  
Press. Cold Spring Harbor, NY (1995); Rosok et al., J.  
25 Biol. Chem. (271:22611-22613 (1996)). Using these  
15 procedures, a library was constructed and a functionally  
active humanized anti- $\alpha,\beta$ -inhibitory antibody was  
identified.

30 For the construction of one grafted form of  
LM609, human framework sequences showing the highest  
20 degree of identity to the murine LM609 V region gene  
sequences were selected for receiving the LM609 CDRs.  
Human heavy chain V region M72 'CL (HHC30Q, HC Subgroup  
3, Kabat et al., *supra*) had 88% identity to frameworks 1,  
2 and 3 of LM609 heavy chain and human light chain V  
40 25 region LS1 'CL (HKL312, Kappa subgroup 3, Kabat et al.,  
*supra*) had 79% identity to frameworks 1, 2 and 3 of LM609  
light chain. Murine LM609 CDR sequences, as defined by  
Kabat et al., *supra* were grafted onto the human  
45 frameworks. Residues predicted to be buried that might  
30 affect the structure and therefore the binding properties  
of the original murine combining site were taken into  
consideration when designing possible changes (Singer et  
50 al., *supra*; Padlan, E.A. Mol. Immunol. 28:489-498

(1991)). This analysis of framework residues considered to be important for preserving the specificity and affinity of the combining site revealed only a few differences. For example, in the heavy chain sequence, the predicted buried residues displayed 100% identity. Of particular note is that Arg16 in human heavy chain V region M72 'CL is a relatively uncommon residue among human chains. However, this residue was also found to be present in LM609 V<sub>H</sub> and therefore was retained. Similarly, Arg19 in LM609 is a relatively rare residue among murine heavy chains but it is found to occur in M72 'CL and was therefore retained. In the light chain sequences, two nonidentical buried residues were identified between LM609 and LS1 'CL framework regions at positions 49 and 87. These two positions were therefore incorporated into the grafted antibody library as both human and murine alternatives.

Full-length grafted V region genes were synthesized by PCR using long overlapping oligonucleotides. Light chain oligonucleotides containing mixed amino acid residues at positions 49 and 87 were synthesized as described in Glaser et al. (J. Immunol. 149:3903-3913 (1992)) and as illustrated in the oligonucleotides represented as V<sub>L</sub> oligo3 and V<sub>L</sub> oligo4. (SEQ ID NOS:16 and 17, respectively). All long oligonucleotides were gel purified.

Grafted LM609 heavy and light chain V regions were constructed by mixing 5 overlapping oligonucleotides at equimolar concentrations, in the presence of annealing PCR primers. The heavy chain oligonucleotides map to the following nucleotide positions: V<sub>H</sub> oligonucleotide 1 (V<sub>H</sub> oligo1), nucleotides (nt) 1-84; (SEQ ID NO:9); V<sub>H</sub> oligo2, nt 70-153, (SEQ ID NO:10); V<sub>H</sub> oligo3, nt 138-225 (SEQ ID

5 NO:11); V<sub>H</sub> oligo4, nt 211-291 (SEQ ID NO:12); V<sub>H</sub> oligo5,  
nt 277-351 (SEQ ID NO:13). Similarly, the Vitaxin light  
chain oligonucleotides map to the following nucleotide  
10 positions: V<sub>L</sub> oligonucleotide 1 (V<sub>L</sub> oligo1), nucleotides  
5 (nt) 1-87; (SEQ ID NO:14); V<sub>L</sub> oligo2, nt 73-144, (SEQ ID  
NO:15); V<sub>L</sub> oligo3, nt 130-213 (SEQ ID NO:16); V<sub>L</sub> oligo4,  
nt 199-279 (SEQ ID NO:17); V<sub>L</sub> oligo5, nt 265-321 (SEQ ID  
15 NO:18). The nucleotide sequences of oligonucleotides  
used to construct grafted LM609 heavy and light chain  
20 variable regions are shown in Table 6. Codon positions  
49 and 87 in V<sub>L</sub> oligo3, and V<sub>L</sub> oligo4 represent the  
randomized codons. The annealing primers contained at  
least 18 nucleotide residues complementary to vector  
sequences for efficient annealing of the amplified V  
25 region product to the single-stranded vector. The  
annealed mixture was fully converted to a double-stranded  
molecule with T4 DNA polymerase plus dNTPs and ligated  
with T4 ligase.

30 To generate the library, a portion of the  
mutagenesis reaction (1  $\mu$ l) was electroporated into *E.*  
20 *coli* strain DH10B (BRL), titered onto a lawn of XL-1  
(Stratagene, Inc.) and incubated until plaques formed.  
35 Replica filter lifts were prepared and plaques containing  
V<sub>H</sub> gene sequences were screened either by hybridization  
25 with a digoxigenin-labeled oligonucleotide complementary  
to LM609 heavy chain CDR 2 sequences or reactivity with  
40 7F11-alkaline phosphatase conjugate, a monoclonal  
antibody raised against the decapeptide sequence Tyr Pro  
Tyr Asp Val Pro Asp Tyr Ala Ser (SEQ ID NO:28) appended  
45 30 to the carboxy terminus of the vector CH<sub>1</sub> domain (Biosite,  
Inc., San Diego, CA). Fifty clones that were double-  
positive were pooled and used to prepare uridinylated  
50 template for hybridization mutagenesis with the amplified  
grafted LM609 V<sub>L</sub> product.

**Table 6: Oligonucleotides Used to Construct Grafted  
LM609 Heavy and Light Chain Variable Regions**

	CAGGTGCAGC	TGGTGGAGTC	TGGGGGAGGC	GTTGTGCAGC	CTGGAAGGTC	
10	CCTGAGACTC	TCCTGTGCAG	CCTCTGGATT	CACC	SEQ ID NO: 9	
5	AACTTTTGCG	ACCCACTCCA	GACCCTTGCC	CGGAGCCTGG	CGAACCCAAG	
	ACATGTCATA	GCTACTGAAG	GTGAATCCAG	AGGC	SEQ ID NO: 10	
	TGGGTCGCAA	AAGTTAGTAG	TGGTGGTGGT	AGCACCTACT	ATTAGACAC	
15	TGTGCAGGGC	CGATTCACCA	TCTCCAGAGA	CAATAGT	SEQ ID NO: 11	
	TGCACAGTAA	TACACGGCTG	TGTCCTCGGC	TCTCAGAGAG	TTCATTTGCA	
10	GGTATAGGGT	GTTCTTACTA	TTGTCTCTGG	A	SEQ ID NO: 12	
	GTGTATTACT	GTGCAAGACA	TAACACGGC	AGTTTGGCTT	ACTGGGGCCA	
20	AGGGACTACA	GTGACTGTTT	CTAGT		SEQ ID NO: 13	
	GAGATTGTGC	TAACACAGTC	TCCAGCCACC	CTGTCTCTCA	GCCAGGAGA	
	AAGGGCGACT	CTTTCCTGCC	AGGCCAGCCA	AAGTATT	SEQ ID NO: 14	
25	GATGAGAAGC	CTTGGGGCTT	GACCAGGCCT	TTGTTGATAC	CAGTGTAGGT	
	GGTTGCTAAT	ACTTTGGCTG	GC		SEQ ID NO: 15	
	CCAAGGCTTC	TCATCWAATA	TCGTTCCAG	TCCATCTCTG	GGATCCCCGC	
	CAGGTTCACT	GGCAGTGGAT	CAGGGACAGA	TTTC	SEQ ID NO: 16	
30	GCTGCCACTC	TGTTGACAGW	AATAGACTGC	AAAATCTTCA	GGCTCCAGAC	
20	TGGAGATAGT	GAGGGTGAAA	TCTGTCCCTG	A	SEQ ID NO: 17	
	CAACAGAGTG	GCAGCTGGCC	TCACACGTTT	GGAGGGGGGA	CCAAGGTGGA	
	AATTAAG				SEQ ID NO: 18	

The mutagenesis reaction was performed as described above with the  $V_H$  oligonucleotides except that the  $V_L$  oligonucleotides 1 to 5 were employed (SEQ ID NOS:14 to 18, respectively). The reaction was electroporated into *E. coli* strain DH10B and filter lifts probed with either goat anti-human kappa chain antibody conjugated to alkaline phosphatase or a goat anti-human Fab antibody using an alkaline phosphatase conjugated rabbit anti-goat secondary reagent for detection. Positive clones co-expressing both  $V_H$  and  $V_L$  gene sequences were selected (160 total) and used to infect *E. coli* strain MK30-3 for preparing soluble Fab fragments.

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The soluble Fab fragments were screened for binding to  $\alpha_v\beta_3$  in an ELISA assay. Four clones that were shown from the ELISA to strongly bind  $\alpha_v\beta_3$  were identified and further characterized. These clones were termed huLM609M13-34, 54, 55 and 145. All four clones were plaque purified and three independent subclones from each clone was used to prepare Fab fragments for additional binding analysis to  $\alpha_v\beta_3$  by ELISA.

20

25

30

In this additional ELISA, duplicate plates were coated with  $\alpha_v\beta_3$  ligand and incubated with the huLM609 periplasmic samples. In one plate, bound huLM609 Fab was detected with goat anti-human kappa chain antibody conjugated to alkaline phosphatase and in the other plate bound huLM609 Fab was detected with 7F11-alkaline phosphatase conjugate, the monoclonal antibody recognizing the decapeptide tag. Subclones huLM609M13-34-1, 2 and 3 and huLM609M13-145-1, 2 and 3 all yielded double positive signals indicating that the Fabs contain functional  $V_H$  and  $V_L$  polypeptides. These results were confirmed in an ELISA assay on M21 cells, a cell line that expresses the integrin  $\alpha_v\beta_3$ .

35

40

45

50

DNA sequence analysis of subclones huLM609M13-34-3 and huLM609M13-145-3 revealed mutations introduced into the library by errors due to oligonucleotide synthesis or by errors arising during PCR amplification. These mutations were corrected in clone huLM609M13-34-3 by site-directed mutagenesis. In the light chain sequence the following corrections were made: His36 to Tyr36 and Lys18 to Arg18. In the heavy chain sequence the following corrections were made: Glu1 to Gln1, Asn3 to Gln3, Leu11 to Val11. Additionally, during the construction of LM609 grafted molecules, residue 28 from

55



5 the heavy chain was considered to be a non-critical  
framework residue and the human residue (Thr28) was  
retained. Subsequently, however, it has been determined  
10 that residue 28 can be considered part of the CDR.  
5 Therefore, residue 28 was converted to the corresponding  
mouse residue at that position (Ala28) using site  
directed mutagenesis with the oligonucleotide 5'-GCT ACT  
15 GAA GGC GAA TCC AGA G-3' (SEQ ID NO:29). This change was  
later determined to not provide benefit over the human  
20 framework threonine at this site, and the threonine was  
retained. The final grafted LM609 clone was designated  
huLM609M13 1135-4 and is termed herein Vitaxin. The DNA  
sequence of clone Vitaxin is shown in Figures 2A and 2B.

### EXAMPLE III

#### Functional Characterization of Vitaxin

30 This Example shows the characterization of  
Vitaxin's binding specificity, affinity and functional  
activity in a number of *in vitro* binding and cell  
adhesion assays.

35 20 The binding specificity of Vitaxin for the  
integrin  $\alpha_v\beta_3$  was initially assessed by measuring binding  
to  $\alpha_v\beta_3$  and its crossreactivity to other  $\alpha_v$ - or  $\beta_3$ -  
containing integrins. Specifically, binding specificity  
40 was assessed by measuring binding to  $\alpha_{IIb}\beta_3$ , the major  
25 integrin expressed on platelets, and to  $\alpha_v\beta_5$ , an integrin  
found prevalent on endothelial cells and connective  
tissue cell types.

45 Briefly, to determine crossreactivity,  
integrins were coated onto an ELISA plate and a series of  
30 antibody dilutions were measured for Vitaxin binding  
50 activity against  $\alpha_v\beta_3$  and the other integrins. The

integrins  $\alpha_v\beta_3$  and  $\alpha_v\beta_5$  were isolated by affinity chromatography as described by Cheresh (1987), *supra*, and Cheresh and Spiro (1987), *supra*.  $\alpha_{IIB}\beta_3$  was purchased from CalBiochem. Briefly, an LM609 affinity column (Cheresh and Spiro (1987), *supra*) was used to isolate  $\alpha_v\beta_3$  from an octylglucoside human placental lysate, whereas an anti- $\alpha_v$  affinity column was used to isolate  $\alpha_v\beta_5$  from the  $\alpha_v\beta_3$ -depleted column flow through. Antibody binding activity was assessed by ELISA using a goat anti-human IgG-alkaline phosphatase conjugate. As a control, a purified human IgG<sub>1</sub> antibody was used since Vitaxin contains a human IgG<sub>1</sub> backbone.

The results of this assay are shown in Figure 4A and reveal that Vitaxin specifically binds to  $\alpha_v\beta_3$  with high affinity. There was no detectable binding to the other  $\alpha_v$ - or  $\beta_3$ -containing integrins at antibody concentrations over 1.0 mg/ml.

In a further series of binding studies, the binding affinity and specificity was assessed in a competitive binding assay with the parental LM609 antibody against  $\alpha_v\beta_3$ . Competitive binding was measured in an ELISA assay as described above with LM609 being the labeled antibody. Binding of LM609 was determined in the presence of increasing concentrations of Vitaxin competitor. Alternatively, the control competitor antibody was again a human IgG<sub>1</sub>.

The results of this competition are presented in Figure 4B and show that specific inhibition of LM609 binding can be observed at Vitaxin concentrations of over 0.1  $\mu$ g/ml. Almost complete inhibition is observed at Vitaxin concentrations greater than 100  $\mu$ g/ml. This level of competitive inhibition indicates that the

parental monoclonal antibody LM609 and the grafted version Vitaxin exhibit essentially identical specificity.

Binding affinity and specificity were also assessed by measuring the inhibitory activity of Vitaxin on  $\alpha_v\beta_3$  binding to fibrinogen. For these studies,  $\alpha_v\beta_3$  was plated onto ELISA plates as described above for the Vitaxin/ $\alpha_v\beta_3$  binding studies. Inhibitory activity of Vitaxin was determined by measuring the amount of bound biotinylated fibrinogen in the presence of increasing concentrations of Vitaxin or control antibody. Briefly, fibrinogen was purchased from CalBiochem and biotinylated with N-hydroxysuccinimidobiotin as described by the manufacturer (Pierce Life Science and Analytical Research). Streptavidin alkaline phosphatase was used to detect the bound fibrinogen.

The results of this assay are presented in Figure 4C and reveal a specific binding inhibition at Vitaxin concentrations higher than about 0.1  $\mu\text{g/ml}$ . These results, combined with those presented above showing specific binding of Vitaxin to  $\alpha_v\beta_3$  and competitive inhibition of LM609, demonstrate that Vitaxin maintains essentially all of the binding characteristics and specificity exhibited by the parental murine monoclonal antibody LM609. Described below are additional functional studies which corroborate these conclusions based on *in vitro* binding assays.

Additional functional studies were performed to further assess the specificity of Vitaxin binding. These studies were directed to the inhibition of integrin  $\alpha_v\beta_3$  binding in cell adhesion assays. Endothelial cell adhesion events are an important component in the

angiogenic process and inhibition of  $\alpha_v\beta_3$  is known to reduce the neovascularization of tumors and thereby reduce the rate of tumor growth. The inhibition of  $\alpha_v\beta_3$ -mediated cell attachment by Vitaxin in these assays is indicative of the inhibitory activity expected when this antibody is used *in situ* or *in vivo*.

Briefly,  $\alpha_v\beta_3$ -positive M21 melanoma cells grown in RPMI containing 10% FBS were used for these cell binding assays. Cells were released from the culture dish by trypsinization and re-suspended in adhesion buffer at a concentration of  $4 \times 10^5$  cells/ml (see below). Vitaxin, LM609 or purified human IgG<sub>1</sub> (control antibody), were diluted to the desired concentration in 250  $\mu$ l adhesion buffer (10 mM Hepes, 2 mM MgCl<sub>2</sub>, 2 mM CaCl<sub>2</sub>, 0.2 mM MnCl<sub>2</sub>, and 1% BSA in Hepes buffered saline at pH 7.4) and added to wells of a 48-well plate precoated with fibrinogen. The fibrinogen was isolated as described above. Each well was coated with 200  $\mu$ l fibrinogen at a concentration of 10  $\mu$ g/ml for 1 hour at 37°C. For the assay, an equal volume of cells (250  $\mu$ l) containing Vitaxin, LM609 or isotype matched control antibody was added to each of the wells, mixed by gentle shaking and incubated for 20 minutes at 37°C. Unbound cells were removed by washing with adhesion buffer until no cells remained in control wells coated with BSA alone. Bound cells were visualized by staining with crystal violet which was subsequently extracted with 100  $\mu$ l acetic acid (10%) and quantitated by determining the absorbance of the solubilized dye at 560 nm.

The results of this assay are shown in Figure 5A and reveal that both Vitaxin and parental antibody LM609 inhibit M21 cell adhesion to fibrinogen over the same concentration range. The inhibitory concentration

for 50% maximal adhesion was calculated to be about 50 ng/ml. Specificity of Vitaxin was shown by the lack of inhibition observed by the control IgG<sub>1</sub> antibody.

In addition to the above cell adhesion results, the inhibitory activity of Vitaxin was also tested in an endothelial cell migration assay. In this regard, the transwell cell migration assay was used to assess the ability of Vitaxin to inhibit endothelial cell migration (Choi et al., J. Vascular Surg., 19:125-134 (1994) and Leavesly et al., J. Cell Biol., 121:163-170 (1993)).

Briefly, human umbilical vein endothelial cells in log phase and at low passage number were harvested by gentle trypsinization, washed and resuspended at a concentration of  $2 \times 10^6$  cells/ml in 37°C HBS containing 1% BSA (20 mM HEPES, 150 mM NaCl, 1.8 mM CaCl<sub>2</sub>, 1.8 mM MgCl<sub>2</sub>, 5 mM KCl, and 5 mM glucose, pH 7.4). Antibodies (Vitaxin, LM609, and IgG<sub>1</sub> control) were diluted to 10 µg/ml from stock solutions. Antibodies were added to cells in a 1:1 dilution (final concentration of antibodies = 5 µg/ml; final concentration of cells =  $1 \times 10^6$  cells/ml) and incubated on ice for 10 - 30 minutes. The cell/antibody suspensions (200 µl to each compartment) were then added to the upper compartments of a Transwell cell culture chamber (Corning Costar), the lower compartments of which had been coated with 0.5 ml of 10 µg/ml vitronectin (in HBS). Vitronectin serves as the chemoattractant for the endothelial cells. The chambers were placed at 37°C for 4 hours to allow cell migration to occur.

Visualization of cell migration was performed by first removing the remaining cells in the upper compartment with a cotton swab. Cells that had migrated

5 to the lower side of the insert were stained with crystal  
violet for 30 minutes, followed by solubilization in  
acetic acid and the absorbance of the dye was measured at  
10 a wavelength of 550 nm. The amount of absorbance is  
5 directly proportional to the number of cells that have  
migrated from the upper to the lower chamber. The  
results of the assay are presented in Figure 7B. Both  
15 Vitaxin and the parental antibody LM609 yielded  
essentially identical inhibitory results. Specifically,  
10 Vitaxin and LM609 inhibited about 60% of the vitronectin-  
induced migration of endothelial cells compared to the  
20 IgG<sub>1</sub> control and to a sample with no inhibitor.

#### EXAMPLE IV

##### Vitaxin-Mediated Inhibition of $\alpha_v\beta_3$ In Animal Models

15 This Example describes the inhibition of tumor  
growth by Vitaxin in two animal models. Tumor growth was  
inhibited by inhibiting at least  $\alpha_v\beta_3$ -mediated  
30 neovascularization with Vitaxin.

The first model measures angiogenesis in the  
35 chick chorioallantoic membrane (CAM). This assay is a  
well recognized model for *in vivo* angiogenesis because  
the neovascularization of whole tissue is occurring.  
Specifically, the assay measures growth factor induced  
40 angiogenesis of chicken CAM vessels growing toward the  
25 growth factor-impregnated filter disk or into the tissue  
grown on the CAM. Inhibition of neovascularization is  
based on the amount and extent of new vessel growth or on  
45 the growth inhibition of tissue on the CAM. The assay  
has been described in detail by others and has been used  
30 to measure neovascularization as well as the  
neovascularization of tumor tissue (Ausprunk et al., Am.  
50 J. Pathol., 79:597-618 (1975); Ossonski et al. Cancer

5                    Res., 40:2300-2309 (1980); Brooks et al. Science,  
264:569-571 (1994a) and Brooks et al. Cell, 79:1157-1164  
10                    (1994b).

15                    Briefly, for growth factor induced angiogenesis  
5 filter disks are punched from #1 Whatman Qualitative  
Circles using a skin biopsy punch. Disks are first  
sterilized by exposure to UV light and then saturated  
with varying concentrations of TNF- $\alpha$  or HBSS as a  
negative control (for at least 1 hour) under sterile  
10 conditions. Angiogenesis is induced by placing the  
saturated filter disks on the CAMs.  
20

25                    Inhibition of angiogenesis is performed by  
treating the embryos with various amounts of Vitaxin and  
controls (antibody or purified human IgG<sub>1</sub>). The  
15 treatments are performed by intravenous injection  
approximately 24 hours after disk placement. After 48  
hours, CAMs are dissected and angiogenesis is scored on a  
scale of 1-4. HBSS saturated filter disks are used as  
the negative control, representing angiogenesis that may  
20 occur in response to tissue injury in preparing CAMs,  
and, values for these CAMs are subtracted out as  
background. Purified human IgG<sub>1</sub> is used as the negative  
control for injections since Vitaxin is of the human IgG<sub>1</sub>  
subclass. Vitaxin was found to inhibit TNF- $\alpha$  induced  
30 angiogenesis in a dose dependent manner. Maximal  
inhibition occurred with a single dose of Vitaxin at 300  
 $\mu$ g which resulted in greater than 80% inhibition compared  
to the human IgG<sub>1</sub> control.  
45

50                    In addition to the above described CAM assay  
30 using growth factor-induced neovascularization,  
additional studies were performed utilizing tumor-induced  
neovascularization. For these assays, angiogenesis was  
55

5 induced by transplanting of  $\alpha_v\beta_3$ -negative tumor  
fragments into the CAMs. The use of  $\alpha_v\beta_3$ -negative tumor  
fragments ensures that any inhibition of tumor growth is  
10 due to the inhibition of  $\alpha_v\beta_3$ -mediated neovascularization  
5 by CAM-derived endothelial cells and not to adhesion  
events mediated by  $\alpha_v\beta_3$  present on the tumor cells.

15 Inhibition of tumor growth was assessed by  
placing a single cell suspension of FG ( $8 \times 10^6$  cells,  
pancreatic carcinoma) and HEP-3 cells ( $5 \times 10^5$  cells,  
10 laryngeal carcinoma) onto CAMs in 30  $\mu$ l. One week later,  
tumors are removed and cut into approximately 50 mg  
20 fragments at which time they are placed onto new CAMs.  
After 24 hours of this second placement embryos are  
injected intravenously with Vitaxin or human IgG<sub>1</sub> as a  
25 negative control. The tumors are allowed to grow for  
about 7 days following which they are removed and  
weighed.

30 The results of Vitaxin treatment on the  
neovascularization of tumors is shown in Figure 6A. The  
20 data is expressed as a mean change in tumor weight and  
demonstrate that Vitaxin is able to inhibit the growth of  
35  $\alpha_v\beta_3$ -negative tumors such as FG and HEP-3 tumor fragments.  
More specifically, there was a mean weight change for  
Vitaxin treated FG tumor fragments of -5.38 whereas a  
40 change of -11.0 was observed for Vitaxin treated HEP-3  
25 tumors. The IgG<sub>1</sub> controls exhibited positive mean weight  
changes of 25.29 and 28.5 for the FG and HEP-3 tumor  
fragments, respectively. These results were obtained  
45 following a single intravenous injection.

30 In a second animal model, the inhibition of Vx2  
carcinoma cells in rabbits was used as a measure of  
50 Vitaxin's inhibitory effect on tumors. The Vx2 carcinoma



5 is a transplantable carcinoma derived from a Shope  
virus-induced papilloma. It was first described in 1940  
and has since been used extensively in studies on tumor  
10 invasion, tumor-host interactions and angiogenesis. The  
5 Vx2 carcinoma is fibrotic in nature, highly aggressive,  
and exhibits features of an anaplastic type carcinoma.  
Propagation of Vx2 tumor is accomplished through serial  
15 transplantation in donor rabbits. Following subcutaneous  
transplantation, it has been reported that after an  
20 initial inflammatory reaction, host repair mechanisms set  
in between days 2 and 4. This repair mechanism is  
characterized by the formation of new connective tissue  
and the production of new capillaries. The newly formed  
25 capillaries are restricted to the repair zone at day 4,  
however, by day 8 they have extended to the outer region  
of the tumor. These characteristics and the  
pharmacokinetics of Vitaxin in rabbits were used to  
determine initial doses and scheduling of treatments for  
30 these experiments. The elimination half life of Vitaxin  
20 in animal serum dosed at 1, 5, and 10 mg/kg was found to  
be 38.9, 60.3, and 52.1 hours, respectively.

35 Growth of Vx2 tumors in the above animal model  
was used to study the effect of Vitaxin after early  
administration on primary tumor growth in rabbits  
25 implanted subcutaneously with Vx2 carcinoma. Briefly,  
Vx2 tumors (50 mg) were transplanted into the inner thigh  
40 of rabbits through an incision between the skin and  
muscle. Measurements of the primary tumor were taken  
throughout the experiment through day 25. At day 28  
45 30 after the transplantation animals were sacrificed and  
tumors were excised and weighed. By day 28, tumors  
became extremely irregular in shape and as a result,  
50 measurements became difficult and were not reflective of

5

tumor volume. Therefore measurements were assessed only through day 25.

10

In a first study, rabbits were treated starting at day 1 post tumor implantation with 5 and 1 mg/kg Vitaxin every four days for 28 days for a total of 7 doses). In both groups, inhibition of tumor growth was observed. In a second series of studies, rabbits were treated beginning at day 7 post tumor implantation as described above for a total of 5 doses. Inhibition of tumor growth was also observed.

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It should be noted that administering a grafted antibody as a repeat dose treatment to rabbits might generate an immune response that can have a neutralizing effect on Vitaxin thus potentially comprising efficacy. Preliminary data suggest that approximately 25-50% of the animals develop such a response.

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The results of each of the Vitaxin treatments described above is shown in Figure 6B and 6C. In the rabbits receiving treatments on day 1, inhibition of tumor growth was observed in both the 1 mg/kg and the 5 mg/kg dosing groups compared to the control PBS treated control. Specifically, a growth inhibition of about 67 and 80% was observed, respectively, as measured by the mean tumor weight. A lesser degree of inhibition was observed in animals that began Vitaxin treatment on day 7 post implantation. These results are shown in Figure 6C. In all cases, inhibition of tumor growth was not seen at Vitaxin concentrations lower than 0.2 mg/kg.

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## EXAMPLE V

Construction of LM609 Grafted Functional Antibody  
Fragments

This Example shows the construction of functional LM609 grafted antibody fragments in which only the CDRs have been transferred from the LM609 donor antibody to a human acceptor framework.

CDR grafting of LM609 to produce a functional antibody fragment was accomplished by the methods set forth below. These procedures are applicable for the CDR grafting of essentially any donor antibody where amino acid residues outside of the CDRs from the donor antibody are not desired in the final grafted product.

Briefly, the protein sequence of the LM609 antibody, was determined by cloning and sequencing the cDNA that encodes the variable regions of the heavy and light chains as described in Example I. The CDRs from the LM609 donor antibody were identified and grafted into homologous human variable regions of a human acceptor framework. Identification of CDR regions were based on the combination of definitions published by Kabat et al., and MacCallum et al.

The boundaries of the CDR regions have been cumulatively defined by the above two publications and are residues 30-35, 47-66 and 97-106 for CDRs 1, 2 and 3, respectively, of the heavy chain variable region and residues 24-36, 46-56, and 89-97 for CDRs 1, 2 and 3, respectively, of the light chain variable region. Non-identical donor residues within these boundaries but outside of CDRs as defined by Kabat et al. were identified and were not substituted into the acceptor

framework. Instead, functional non-donor amino acid residues were identified and substituted for certain of these non-identical residues.

As described below, the only non-identical residue outside of the CDRs as defined by Kabat et al. but within the CDRs as defined above is at position 49 of the LM609 light chain. To identify functional non-donor amino acids at this position, a library of nineteen antibodies was constructed that contained all non-donor amino acids at position 49 and then screened for binding activity against  $\alpha\beta_3$ .

Human immunoglobulin sequences were identified from the Brookhaven Protein Data Bank-Kabat Sequences of Proteins of Immunological Interest database (release 5.0). Human framework sequences showing significant identity to the murine LM609 variable region gene sequences were selected for receiving the LM609 CDRs. Human heavy chain variable region M72 'CL had 88% identity to frameworks 1, 2 and 3 of LM609 heavy chain and human light chain V region LS1 'CL had 79% identity to frameworks 1, 2 and 3 of LM609 light chain. With the exclusion of non-identical residues outside of the CDRs as defined by Kabat et al. murine LM609 CDR sequences as defined by Kabat et al. and MacCallum et al. were grafted onto the human frameworks. Using this grafting scheme, the final grafted product does not contain any amino acid residues outside of the CDRs as defined by Kabat et al. which are identical to an LM609 amino acid at the corresponding position (outside of residues: 31-35, 50-66 and 99-106 for CDRs 1, 2 and 3, respectively, of the heavy chain variable region and residues 24-34, 50-56, and 89-97 for CDRs 1, 2 and 3, respectively, of the light chain variable region). Moreover, no intermediates are

produced which contain an amino acid residue outside of the CDRs as defined by Kabat et al. which are identical to the LM609 amino acid at that position. The CDR grafting procedures are set forth below.

Full-length CDR grafted variable region genes were synthesized by PCR using long overlapping oligonucleotides as described previously in Example II. The heavy chain variable region oligonucleotides were those described previously as SEQ ID NOS:9-13. The light chain variable region oligonucleotides were synthesized so as to contain the CDR grafted variable region as well as a stop codon at position 49. The five oligonucleotides for the light chain LM609 grafted variable region are shown as SEQ ID NOS:23-27 where the second oligonucleotide in the series contains the stop codon at position 49 (SEQ ID NO:24). The nucleotide sequences of oligonucleotides used to construct LM609 grafted light chain variable region is shown in Table 7.

**Table 7: Oligonucleotides Used to Construct LM609**

**Grafted Light Chain Variable Region**

GAGATTGTGC	TAACTCAGTC	TCCAGCCACC	CTGTCTCTCA	GCCCAGGAGA	
AAGGGCGACT	CTTTCCTGCC	AGGCCAGCCA	AAGTATT	SEQ ID NO: 23	
TTAGATGAGA	AGCCTTGGGG	CTTGACCAGG	CCTTTGTTGA	TACCAGTGTA	
GGTGGTTGCT	AATACTTTGG	CTGGC		SEQ ID NO: 24	
CCAAGGCTTC	TCATCTAATA	TCGTTCCCAG	TCCATCTCTG	GGATCCCCGC	
CAGGTTCACT	GGCAGTGGAT	CAGGGACAGA	TTTC	SEQ ID NO: 25	
GCTGCCACTC	TGTTGACAGT	AATAGACTGC	AAAATCTTCA	GGCTCCAGAC	
TGGAGATAGT	GAGGGTGAAA	TCTGTCCCTG	A	SEQ ID NO: 26	
CAACAGAGTG	GCAGCTGGCC	TCACACGTTT	GGAGGGGGGA	CCAAGGTGGA	
AATTAAG				SEQ ID NO: 27	

5 All long oligonucleotides were gel purified.  
CDR grafting of the LM609 heavy chain variable region was  
constructed by mixing 5 overlapping oligonucleotides (SEQ  
10 ID NOS:9-13), at equimolar concentrations, in the  
5 presence of annealing PCR primers containing at least 18  
nucleotide residues complementary to vector sequences for  
the efficient annealing of the amplified V region product  
15 to the single-stranded vector. The annealed mixture was  
fully converted to a double-stranded molecule with T4 DNA  
10 polymerase plus dNTPs and ligated with T4 ligase. The  
mutagenesis reaction (1  $\mu$ l) was electroporated into *E.*  
20 *coli* strain DH10B (BRL), titered onto a lawn of XL-1  
(Stratagene, Inc.) and incubated until plaques formed.  
Replica filter lifts were prepared and plaques containing  
25  $V_H$  gene sequences were screened either by hybridization  
with a digoxigenin-labeled oligonucleotide complementary  
to LM609 heavy chain CDR 2 sequences or reactivity with  
7F11-alkaline phosphatase conjugate, a monoclonal  
30 antibody raised against the decapeptide sequence Tyr Pro  
20 Tyr Asp Val Pro Asp Tyr Ala Ser (SEQ ID NO:28) appended  
to the carboxy terminus of the vector  $CH_1$  domain (Biosite,  
Inc., San Diego, CA).

35 Fifty clones that were double-positive were  
pooled and used to prepare uridinylated template for  
25 hybridization mutagenesis with the amplified CDR grafted  
LM609  $V_L$  product constructed in a similar fashion using  
40 the five overlapping oligonucleotides shown as SEQ ID  
NOS:23-27. The mutagenesis reaction was electroporated  
into *E. coli* strain DH10B. Randomly picked clones were  
45 30 sequenced to identify a properly constructed template for  
construction of the non-donor library at position 49.  
This template was prepared as a uridinylated template and  
50 an oligonucleotide population of the following sequence  
was used for site directed mutagenesis.

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GGGAACGATA-19aa-GATGAGAAGC

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The sequence 19aa in the above primer (SEQ ID NO:30) represents the fact that this primer specifies a sequence population consisting of 19 different codon sequences that encode each of the 19 non-donor amino acids. These amino acids are those not found at position 49 of LM609 and include all amino acids except for Lys. Clones that resulted from this mutagenesis were picked and antibody expressed by these clones were prepared. These samples were then screened for binding to  $\alpha\beta$ , in an ELISA assay. Clones having either Arg or Met amino acids in position 49 were functionally identified. The nucleotide and amino acid sequence of the LM609 grafted heavy chain variable region is shown in Figure 1A (SEQ ID NOS:1 and 2, respectively). The nucleotide and amino acid sequence of the LM609 grafted light chain variable region is shown in Figure 7 (SEQ ID NOS:31 and 32, respectively).

#### EXAMPLE VI

##### Generation of LM609 Grafted Antibodies Having Enhanced Activity

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This example shows *in vitro* maturation of LM609 grafted antibody to obtain antibody variants having increased affinity to  $\alpha\beta$ , relative to the parent LM609 grafted antibody.

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To optimize the affinity of LM609 grafted antibody *in vitro*, an M13 phage system was used, which permits the efficient synthesis, expression, and screening of libraries of functional antibody fragments (Fabs). The contribution of each of the six CDRs of the Ig heavy and light chains was assessed. The CDRs were

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5 defined broadly based on a combination of sequence  
variability and antibody structural models (Kabat et al.,  
10 J. Biol. Chem. 252:6609-6616 (1977); Chothia et al.,  
*supra*; MacCallum et al., *supra*). Thus, one library was  
5 constructed for each CDR, with the exception of H2 which  
was split into two libraries due to its long (20 amino  
15 acids) length. The variable region frameworks which  
harbored the mutated CDRs were the heavy chain variable  
region shown in Figure 1a (SEQ ID NO:2) and the light  
10 chain variable region shown in Figure 7 (SEQ ID NO:32).

20 CDRs were chosen from the heavy chain variable  
region shown in Figure 1a (SEQ ID NO:2) and the light  
chain variable region shown in Figure 7 (SEQ ID NO:32).  
25 Briefly, utilizing the numbering system of Kabat et al.,  
*supra*, the residues chosen for mutagenesis of the CDRs  
(Table 9) were: Gln<sup>24</sup>-Tyr<sup>36</sup> in light chain CDR1 (L1);  
Leu<sup>46</sup>-Ser<sup>56</sup> in light chain CDR2 (L2); Gln<sup>89</sup>-Thr<sup>97</sup> in light  
30 chain CDR3 (L3); Gly<sup>26</sup>-Ser<sup>35</sup> in heavy chain CDR1 (H1);  
Trp<sup>47</sup>-Gly<sup>65</sup> in heavy chain CDR2 (H2); and Ala<sup>93</sup>-Tyr<sup>102</sup> in  
20 heavy chain CDR3 (H3). Libraries were created for each  
CDR, with the oligonucleotides designed to mutate a  
35 single CDR residue in each clone. Due to the extended  
length of H2, two libraries mutating residues 47-55 (H2a)  
and 56-65 (H2b), respectively, were constructed to cover  
25 this region.

40 The template for generating light chain CDR3  
mutants contained Gly at position 92. However, it was  
subsequently determined that position 92 of the light  
45 chain CDR3 was inadvertently deduced to be a Gly,  
30 resulting in humanized LM609 grafted antibodies being  
constructed with Gly at that position. It was later  
realized that the original LM609 sequence contained an  
50 Asn at position 92. Using the methods described herein



to introduce mutations into CDRs of an LM609 grafted antibody, an LM609 grafted antibody having Asn at position 92 of light chain CDR3 was found to have  $\alpha_v\beta_3$  binding activity (see Table 9), confirming the identification of Asn<sup>92</sup> as a functional LM609 grafted antibody. Thus, antibodies containing light chain CDR3 having Gly or Asn at position 92 are active in binding  $\alpha_v\beta_3$ .

Oligonucleotides encoding a single mutation were synthesized by introducing NN(G/T) at each CDR position as described previously (Glaser et al., *supra*). The antibody libraries were constructed in M131XL604 vector by hybridization mutagenesis as described previously, with some modifications (Rosok et al., J. Biol. Chem. 271:22611-22618 (1996); Huse et al., J. Immunol. 149:3914-3920 (1992); Kunkel, Proc. Natl. Acad. Sci. USA 82:488-492 (1985); Kunkel et al., Methods Enzymol. 154:367-382 (1987)). Briefly, the oligonucleotides were annealed at a 20:1 molar ratio to uridynylated LM609 grafted antibody template (from which the corresponding CDR had been deleted) by denaturing at 85°C for 5 min, ramping to 55°C for 1 h, holding at 55°C for 5 min, then chilling on ice. The reaction was extended by polymerization and electroporated into DH10B and titered onto a lawn of XL-1 Blue. The libraries consisted of pools of variants, each clone containing a single amino acid alteration in one of the CDR positions. Utilizing codon-based mutagenesis, every position in all of the CDRs was mutated, one at a time, resulting in the subsequent expression of all twenty amino acids at each CDR residue (Glaser et al., *supra*). The CDR libraries ranged in size from 288 (L3) to 416 (L1) unique members and contained a total of 2336 variants.

5 To permit the efficient screening of the  
initial libraries, a highly sensitive plaque lift assay,  
termed capture lift, was employed (Watkins et al., Anal.  
10 Biochem. 256 (1998)). Briefly, phage expression  
5 libraries expressing LM609 grafted antibody variants were  
initially screened by a modified plaque lift approach, in  
which the nitrocellulose was pre-coated with goat  
15 anti-human kappa antibody and blocked with bovine serum  
albumin prior to application to the phage-infected  
20 bacterial lawn. Following the capture of phage-expressed  
LM609 grafted antibody variant Fabs, filters were  
incubated with 1.0  $\mu\text{g/ml}$  biotinylated  $\alpha_v\beta_3$  for 3 h at 4°C,  
washed four times, incubated with 2.3  $\mu\text{g/ml}$   
NeutrAvidin-alkaline phosphatase (Pierce Chemical Co.;  
25 Rockford, IL) for 15 min at 25°C, and washed four times.  
All dilutions and washes were in binding buffer.  
Variants that bound  $\alpha_v\beta_3$  were identified by incubating the  
filters for 10-15 min in 0.1 M Tris, pH 9.5, containing  
30 0.4 mM 2,2'-di-*p*-nitrophenyl-5,5'-diphenyl-3,  
20 3'-(3,3'-dimethoxy-4,4'-diphenylene)ditetrazolium  
chloride and 0.38 mM 5-bromo-4-chloro-3-indoxyl phosphate  
mono-(*p*-toluidinium) salt (JBL Scientific, Inc.; San Luis  
35 Obispo, CA).

To generate biotinylated  $\alpha_v\beta_3$ , the  $\alpha_v\beta_3$  receptor  
25 was purified from human placenta by affinity  
chromatography, as described previously (Smith and  
40 Cheresh, J. Biol. Chem. 263:18726-18731 (1988)). To  
biotinylate  $\alpha_v\beta_3$ , purified receptor was dialyzed into 50  
mM HEPES, pH 7.4, 150 mM NaCl, 1.0 mM  $\text{CaCl}_2$ , containing  
45 30 0.1% NP-40 (binding buffer) and incubated with 100-fold  
molar excess sulfosuccinimidobiotin for 3h at 4°C. The  
reaction was terminated by the addition of 50 mM  
ethanolamine.  
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5 Phage expressed LM609 grafted antibody variants  
were selectively captured on nitrocellulose filters  
coated with goat anti-human kappa chain antibody, probed  
10 with biotinylated  $\alpha_v\beta_3$ , and detected with  
5 NeutrAvidin-alkaline phosphatase. Initially,  
biotinylated  $\alpha_v\beta_3$  was titrated on lifts containing phage  
expressing the LM609 grafted antibody parent molecule  
15 only. Subsequently, the concentration of biotinylated  
 $\alpha_v\beta_3$  was decreased to yield a barely perceptible signal.  
10 In this way, only clones expressing higher affinity  
variants were readily identified during screening of the  
20 variant libraries. Following the exhaustive capture lift  
screening of  $\geq 2500$  clones from each library, 300 higher  
affinity variants were identified (see Table 8). The  
25 greatest number of clones displaying improved affinity  
were identified in the H3 (185) and L3 (52) CDRs, though  
variants with improved affinity were identified in every  
CDR.

30 LM609 grafted antibody variants identified by  
20 capture lift as having  $\alpha_v\beta_3$  binding activity were further  
characterized to determine binding affinity to  $\alpha_v\beta_3$ ,  
35 specificity for  $\alpha_v\beta_3$  over other integrins, and  $\alpha_v\beta_3$   
association and dissociation rates. For these assays,  
purified Fab of LM609 grafted antibody variants was used.  
25 Briefly, Fab was expressed as described previously and  
was released from the periplasmic space by sonic  
40 oscillation (Watkins et al., *supra*, 1997). Cells  
collected from one liter cultures were lysed in 10 ml  
50 mM Tris, pH 8.0, containing 0.05% Tween 20. Fab was  
45 30 bound to a 1 ml protein A column (Pharmacia) which had  
been equilibrated with 50 mM glycine, pH 8, containing  
250 mM NaCl, washed with the same buffer, and eluted with  
50 10 ml of 100 mM glycine, pH 3, into one-tenth volume 1 M

Tris, pH 8. Purified Fab was quantitated as described previously (Watkins et al., *supra*, 1997).

LM609 grafted antibody variants were tested for binding to  $\alpha_v\beta_3$  and specificity of binding to  $\alpha_v\beta_3$  relative to  $\alpha_v\beta_5$  and  $\alpha_{IIB}\beta_3$ . For ELISA titration of Fab on immobilized  $\alpha_v\beta_3$  and the related integrins  $\alpha_v\beta_5$  and  $\alpha_{IIB}\beta_3$ , Immulon II microtiter plates were coated with 1  $\mu$ g/ml purified receptor in 20 mM Tris, pH 7.4, 150 mM NaCl, 2 mM  $\text{CaCl}_2$ , 1 mM  $\text{MgCl}_2$ , 1 mM  $\text{MnCl}_2$ , washed once, and blocked in 3% BSA in 50 mM Tris, pH 7.4, 100 mM NaCl, 2 mM  $\text{CaCl}_2$ , 1 mM  $\text{MgCl}_2$  for 1 h at 25°C. Human  $\alpha_{IIB}\beta_3$ , purified from platelets, was obtained from Enzyme Research Laboratories, Inc. (South Bend, IN) and  $\alpha_v\beta_5$  was purified from placental extract depleted of  $\alpha_v\beta_3$ , as described previously (Smith et al., *J. Biol. Chem.* 265:11008-11013 (1990)). Just prior to use, the plates were washed two times and were then incubated 1 h at 25°C with various dilutions of Fab. The plates were washed five times, incubated 1 h at 25°C with goat anti-human kappa-alkaline phosphatase diluted 2000-fold, washed five times, and developed as described previously (Watkins et al., *supra*, 1997). All dilutions and washes were in 50 mM Tris-HCl, pH 7.4, 100 mM NaCl, 2 mM  $\text{CaCl}_2$ , and 1 mM  $\text{MgCl}_2$ .

**Table 8: Capture Lift Screening of LM609 grafted antibody CDR Libraries.**

Library	Size <sup>1</sup>	Screened <sup>2</sup>	Positives <sup>3</sup>	Enhanced Affinity <sup>4</sup>
H1	320	2500	16	8
H2a	320	5000	26	7
H2b	320	5000	2	1
H3	320	5000	185	78 <sup>5</sup>
L1	416	2500	12	1
L2	352	3250	7	1
L3	288	5000	52	41

<sup>1</sup>Number of unique clones based on DNA sequence. Thirty-two codons are used to express all twenty amino acids at each position.

<sup>2</sup>Phage-expressed libraries were plated on XL-1 Blue/agar lawns at 500-100 plaques per 100 mm dish.

<sup>3</sup>Positives are defined as clones that were identified in the initial screen, replated, and verified in a second capture lift assay.

<sup>4</sup>Soluble Fab was titrated against immobilized  $\alpha_v\beta_3$  in an ELISA format. Based on comparison of the inflection point of the titration profiles, clones which displayed -3-fold enhanced affinity were selected for further characterization.

<sup>5</sup>Of the 185 positive clones identified by capture lift, 98 were further characterized for binding to immobilized  $\alpha_v\beta_3$ .

Figure 8 shows titration of antibody variants and LM609 grafted antibody Fab on immobilized  $\alpha_v\beta_3$ . Bacterial cell lysates containing LM609 grafted antibody (closed circles), variants with improved affinity isolated from the primary libraries (S102, closed squares; Y100, open squares; and Y101, open triangles) or from the combinatorial libraries (closed triangles), or an irrelevant Fab (open circles) were titrated on immobilized  $\alpha_v\beta_3$ .

Comparison of the inflection points of the binding profiles obtained from titrating variants on immobilized  $\alpha_v\beta_3$  demonstrated that multiple clones displayed >3-fold improved affinity, confirming the effectiveness of utilizing the capture lift in a semi-quantitative fashion (Figure 8, compare squares and open triangles with closed circles). Based on the capture lift screening and subsequent characterization of binding to immobilized  $\alpha_v\beta_3$ , it was concluded that both heavy and light chain CDRs are directly involved in the interaction of  $\alpha_v\beta_3$  with the LM609 grafted antibody variants.

DNA was isolated from clones displaying >3-fold enhanced binding and sequenced to identify the mutations which resulted in higher affinity. DNA sequencing was performed on isolated single-stranded DNA. The heavy and light chain variable region genes were sequenced by the fluorescent dideoxynucleotide termination method (Perkin-Elmer; Foster City, CA). Based on sequence analysis of 103 variants, 23 unique mutations clustered at 14 sites were identified (Table 9). The majority of the sites of beneficial mutations were found in the heavy chain CDRs, with four located in H3, and three each in H2 (2a and 2b combined) and H1. Seven distinct and

beneficial amino acid substitutions were identified at a single site within H3, tyrosine residue 102. The diverse nature of the substitutions at this site suggests that tyrosine residue 102 may sterically hinder LM609 grafted antibody binding to  $\alpha_v\beta_3$ . In support of this, variants expressing the other aromatic amino acids (phenylalanine, histidine, and tryptophan) instead of tyrosine at residue 102 were never isolated following screening for enhanced binding.

The affinities of select variants were further characterized by utilizing surface plasmon resonance (BIAcore) to measure the association and dissociation rates of purified Fab with immobilized  $\alpha_v\beta_3$ . Briefly, surface plasmon resonance (BIAcore; Pharmacia) was used to determine the kinetic constants for the interaction between  $\alpha_v\beta_3$  and LM609 grafted antibody variants. Purified  $\alpha_v\beta_3$  receptor was immobilized to a (1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride)/N-hydroxysuccinimide-activated sensor chip by injecting 30  $\mu$ l of 15  $\mu$ g/ml  $\alpha_v\beta_3$  in 10 mM sodium acetate, pH 4. To obtain association rate constants ( $k_{on}$ ), the binding rate at five different Fab concentrations, ranging from 5-40  $\mu$ g/ml in 50 mM Tris-HCl, pH 7.4, 100 mM NaCl, 2 mM CaCl<sub>2</sub>, and 1 mM MgCl<sub>2</sub>, was determined at a flow rate of 10  $\mu$ l/min. Dissociation rate constants ( $k_{off}$ ) were the average of five measurements obtained by analyzing the dissociation phase at an increased flow rate (40  $\mu$ l/min). Sensorgrams were analyzed with the BIAevaluation 2.1 program (Pharmacia). Residual Fab was removed after each measurement with 10 mM HCl, 2 mM CaCl<sub>2</sub> and 1 mM MgCl<sub>2</sub>.

5 Table 9 shows that the variants all displayed a  
lower Kd than the LM609 grafted antibody parent molecule,  
consistent with both the capture lift and the ELISA.  
10 Analysis of association and dissociation rates revealed  
5 that the majority of improved variants had slower  
dissociation rates while having similar association  
rates. For example, LM609 grafted antibody had an  
15 association rate  $18.0 \times 10^4 \text{ M}^{-1}\text{s}^{-1}$ , while the variants  
ranged from  $16.7\text{--}31.8 \times 10^4 \text{ M}^{-1}\text{s}^{-1}$ . In contrast, every  
10 clone dissociated slower than LM609 grafted antibody  
( $4.97 \times 10^{-3} \text{ s}^{-1}$ ) with dissociation rates ranging from  
20 1.6-fold ( $3.03 \times 10^{-3} \text{ s}^{-1}$ ) to 11.8-fold ( $0.42 \times 10^{-3} \text{ s}^{-1}$ )  
slower.

25 These results demonstrate that introducing  
15 single amino acid substitutions into LM609 grafted  
antibody CDRs allows the identification of modified LM609  
grafted antibodies having higher affinity for  $\alpha_v\beta_3$  than  
30 the parent LM609 grafted antibody.



Table 9: Identification of Enhanced LM609 Grafted Antibodies from Primary Libraries

chain	library†	sequence	$k_{on}$ ( $\times 10^4$ ) ( $M^{-1}s^{-1}$ )	$k_{off}$ ( $\times 10^{-3}$ ) ( $s^{-1}$ )	Kd (nM)
	LM609 grafted antibody		18.0	4.97	27.6
H	CDR1 T27 W29- L30	G F T F S S Y D M S T W L	n.d. n.d. n.d.	n.d. n.d. n.d.	n.d. n.d. n.d.
H	CDR2a K52	W V A K V S S G G G K	17.8	2.18	12.2
H	CDR2b P60 E64	S T Y Y L D T V Q G P E	31.8 n.d.	1.85 n.d.	5.8 n.d.
H	CDR3 H97 Y100 D101 Y101 S102 T102 D102 E102 M102 G102 A102	A R H N Y G S F A Y H Y Y D Y S T D E M G A	22.0 17.5 n.d. 21.8 24.2 24.6 27.6 n.d. n.d. 16.1 27.5	3.03 2.51 n.d. 0.48 1.44 1.43 0.97 n.d. n.d. 2.01 2.27	13.8 14.3 n.d. 2.2 6.0 5.8 3.5 n.d. n.d. 12.5 8.3
L	CDR1 F32	Q A S Q S I S N H L H W Y F	16.7	0.42	2.5
L	CDR2 S51	L L I R Y R S Q S I S S	n.d.	n.d.	n.d.
L	CDR3 N92 T92 L96 Q96	Q Q S G S W P P H T N T L Q	23.6 n.d. 24.3 n.d.	1.35 n.d. 2.23 n.d.	5.7 n.d. 9.2 n.d.

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EXAMPLE VIIGeneration of High Affinity LM609 Grafted Antibodies

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This example shows that single amino acid mutations in CDRs of an LM609 grafted that result in higher affinity binding to  $\alpha_v\beta_3$ , can be combined to generate high affinity LM609 grafted antibodies.

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Random combination of all of the beneficial mutations of LM609 grafted antibody would generate a combinatorial library containing  $>10^5$  variants, requiring efficient screening methodologies. Therefore, to determine if clones displaying  $>10$ -fold enhanced affinities could be rapidly distinguished from one another, variants displaying 3 to 13-fold enhanced affinity were evaluated by capture lift utilizing lower concentrations of biotinylated  $\alpha_v\beta_3$ . Despite repeated attempts with a broad range of concentrations of  $\alpha_v\beta_3$ , consistent differences in the capture lift signals were not observed. Because of this, smaller combinatorial libraries were constructed and subsequently screened by ELISA.

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Four distinct combinatorial libraries were constructed in order to evaluate the optimal number of combinations that could be accomplished utilizing two site hybridization mutagenesis (Figure 9). Briefly, combinatorial libraries were constructed by synthesizing degenerate oligonucleotides encoding both the wild-type and beneficial heavy chain mutations (H2, Leu<sup>60</sup>-Pro; H3 Tyr<sup>97</sup>-His; H3, Ala<sup>101</sup>-Tyr; H3, Tyr<sup>102</sup>-Ser, Thr, Asp, Glu, Met, Gly, Ala). Utilizing two site hybridization mutagenesis, as described above, the oligonucleotides were annealed at a 40:1 molar ratio to uridynylated template prepared from LM609 grafted antibody and three

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light chain mutations (Figure 9; L1, His<sup>32</sup>-Phe; L3, Gly<sup>92</sup>-Asn; L3, His<sup>96</sup>-Leu). As a result, a total of 256 variants were synthesized in four combinatorial library subsets.

Figure 9 shows construction of combinatorial libraries of beneficial mutations. Uridinylated template from LM609 grafted antibody and three optimal light chain variants (F32, N92, and L96) was prepared. Two site hybridization was performed with two degenerate oligonucleotides, which were designed to introduce beneficial mutations at four distinct heavy chain residues.

Following preparation of uridinylated templates of LM609 grafted antibody and three light chain variants, (Table 9; F32, N92, and L96), degenerate oligonucleotides encoding the wild type residue and the most beneficial heavy chain mutations (Table 9; P60, H97, Y101, S102, T102, D102, E102, M102, G102, and A102) were hybridized to the light chain templates, resulting in four combinatorial libraries, each containing 64 unique variants. Potentially, the combination of multiple mutations can have detrimental effects on affinity and, thus, can prevent the identification of beneficial combinations resulting from mutations at fewer sites. For this reason, the amino acid expressed by the LM609 grafted antibody parent molecule was included at each position in the combinatorial library. By utilizing this approach, simultaneous combinatorial mutagenesis of three CDRs (L1 or L3 each in combination with H2 and H3) was accomplished. Based on sequence analysis, the two site hybridization mutagenesis was achieved with ~50% efficiency.

5 In order to screen the combinatorial libraries,  
soluble Fab was expressed and released from the periplasm  
of small-scale (<1 ml) bacterial cultures that had been  
10 infected with randomly selected clones. Although  
5 variable expression levels were observed, uniform  
quantities of the unpurified variants were captured on a  
microtiter plate through a peptide tag present on the  
15 carboxyl-terminus of the heavy chain. Briefly,  
combinatorial LM609 grafted antibody libraries were  
10 screened by an ELISA that permits the determination of  
relative affinities of antibody variants produced in  
20 small-scale bacterial cultures (Watkins et al., Anal.  
Biochem. 253:37-45 (1997)). An Immulon II microtiter  
plate (Dynatech Laboratories; Chantilly, VA) was coated  
25 with 10  $\mu$ g/ml of the 7F11 monoclonal antibody, which  
recognizes a peptide tag on the carboxyl-terminus of the  
LM609 grafted antibody variant heavy chains. (Field et  
al., Mol. Cell. Biol. 8:2159-2165 (1988)). Following  
30 capture of Fab from *E. coli* lysates, the plate was  
20 incubated with 0.5-1  $\mu$ g/ml biotinylated  $\alpha_v\beta_3$  for 1 h at  
25°C. The plate was washed seven times, incubated with  
0.5 U/ml streptavidin-alkaline phosphatase (1000 U/ml;  
35 Boehringer Mannheim; Indianapolis, IN) for 15 min at  
25°C, washed seven times, and developed as described  
25 previously (Watkins et al., *supra*, 1997). All dilutions  
and washes were in binding buffer.

40 As described previously (Watkins et al., *supra*,  
1997), this ELISA screening method enabled a rapid and  
direct comparison of the relative affinities of the  
45 30 variants following incubation with biotinylated  $\alpha_v\beta_3$  and  
streptavidin-alkaline phosphatase. To ensure that the  
full Fab diversity was sampled, one thousand randomly  
50 selected clones were screened from each combinatorial  
library. Variants that displayed an enhanced ELISA

5 signal were further characterized for binding to  
immobilized  $\alpha_v\beta_3$  (Figure 8, closed triangles) and were  
10 sequenced to identify the mutations (Table 10).

Screening of the four combinatorial libraries  
5 identified fourteen unique combinations of mutations that  
improved binding significantly over the individual  
15 mutations identified in the screening of the first  
library. While the best clone from the primary screen  
had a 12.5-fold increase in affinity, the fourteen unique  
20 combinations isolated from screening the combinatorial  
libraries displayed affinities ranging from 18 to 92-fold  
greater than the parent LM609 grafted antibody. The  
majority of these variants consisted of H2 and H3  
25 mutations combined with the L1 or L3 mutations.  
15 Beneficial combinations of heavy chain mutations with  
wild-type light chain were also identified, but did not  
result in improved affinity to the same extent as other  
combinatorial variants. The variants predominantly  
30 contained 2 to 4 mutations, with one clone, C29,  
20 containing five mutations. No direct correlation between  
the total number of mutations in each variant and the  
35 resulting affinity was observed. For example, while the  
binding of clone C37 was 92-fold enhanced over the parent  
molecule and was achieved through the combination of  
25 three mutations, clone C29 had ~55-fold greater affinity  
40 achieved through the combination of five mutations.  
Multiple variants displaying >50-fold enhanced affinity  
resulting from the combination of as few as two mutations  
were identified (2G4, 17, and V357D).

45  
30 The combinatorial clones with improved affinity  
all displayed >10-fold slower dissociation rates,  
possibly reflecting a selection bias introduced by long  
50 incubation steps in the screening. In addition, all of

5 the combinatorial variants isolated from the library  
based on the L96 light chain mutation also displayed 2 to  
10 4-fold greater association rates. Previously, it has  
5 been demonstrated that the antibody repertoire shifts  
towards immunoglobulins displaying higher association  
rates during affinity maturation *in vivo* (Foote and  
15 Milstein, Nature 352:530-532 (1991)). The L96 subset of  
variants, therefore, may more closely mimic the *in vivo*  
affinity maturation process where B-lymphocyte  
10 proliferation is subject to a kinetic selection.

20 LM609 grafted antibody binds the  $\alpha_v\beta_3$  complex  
specifically and does not recognize either the  $\alpha_v$  or the  
 $\beta_3$  chain separately. To further characterize the  
25 variants, clones were screened for reactivity with the  
related integrins,  $\alpha_{IIb}\beta_3$  and  $\alpha_v\beta_5$ . All variants tested  
were unreactive with both  $\alpha_{IIb}\beta_3$  and  $\alpha_v\beta_5$ , consistent with  
the improved binding not substantially altering the  
30 interaction of Fab and receptor.

As a first step toward determining if the  
20 increase in affinity of the variants resulted in greater  
biological activity, variants displaying a range of  
35 affinities were assayed for their ability to inhibit the  
binding of a natural ligand, fibrinogen, to immobilized  
 $\alpha_v\beta_3$  receptor. Briefly, LM609 grafted antibody variants  
40 25 were tested for inhibition of ligand binding as described  
previously except that the binding of biotinylated human  
fibrinogen (Calbiochem, La Jolla, CA) was detected with  
0.5  $\mu$ g/ml NeutrAvidin-alkaline phosphatase (Smith et al.,  
45 J. Biol. Chem. 265:12267-12271 (1990)).

Table 10: Identification of Optimal Combinatorial Mutations

library*	clone	L1 32	L3 92	L3 96	L3 96	H2 60	H3 97	H3 101	H3 102	$k_{on}$ ( $\times 10^4$ ) ( $M^{-1}s^{-1}$ )	$k_{off}$ ( $\times 10^{-3}$ ) ( $s^{-1}$ )	Kd (nM)
wild type		H	G	H	H	L	Y	A	Y	18.0	4.97	27.6
F32	17	F							S	25.1	0.138	0.5
	7	F				P	H		S	20.4	0.236	1.2
	56	F				P			S	26.6	0.135	0.5
	C59	F				P			D	26.5	0.137	0.5
	C176	F				P			T	22.5	0.192	0.9
	V357D	F							D	27.9	0.140	0.5
N92	C119		N			P			S	21.5	0.316	1.5
L96	8F9			L	P	P	H		S	47.5	0.280	0.6
	C29			L	P	P	H	Y	S	67.5	0.343	0.5
	264			L					S	60.3	0.229	0.4
	6H6			L			H		S	50.4	0.187	0.4
	C37			L				Y	E	44.8	0.147	0.3
	6D1			L	P	P		Y	S	41.0	0.158	0.4
	6G1			L	P	P			S	38.9	0.280	0.7

5 The results of these competition assays are  
shown in Figure 10. Figure 10A shows inhibition of  
fibrinogen binding to immobilized  $\alpha_v\beta_3$ . Immobilized  $\alpha_v\beta_3$   
10 was incubated with 0.1  $\mu\text{g/ml}$  biotinylated fibrinogen and  
5 various concentrations of LM609 grafted antibody (open  
circles), S102 (closed circles), F32 (open triangles), or  
C59 (closed triangles) for 3 h at 37°C. Unbound ligand  
15 and Fab were removed by washing and bound fibrinogen was  
quantitated following incubation with NeutrAvidin  
20 alkaline phosphatase conjugate. Figure 10B shows  
correlation of affinity of variants with inhibition of  
fibrinogen binding. The concentration of variants  
required to inhibit the binding of fibrinogen to  
25 immobilized  $\alpha_v\beta_3$  by 50% ( $\text{IC}_{50}$ ) was plotted as a function of  
the affinity (Kd).

As shown in Figure 10A, higher affinity  
variants were more effective at blocking the ligand  
30 binding site of the receptor (compare LM609 grafted  
antibody, open circles, with any of the variants).  
20 Subsequent analysis of ten variants displaying affinities  
(Kd) ranging from 0.3 to 27 nM demonstrated a good  
35 correlation ( $r^2 = 0.976$ ) between affinity and ability to  
inhibit fibrinogen binding (Figure 10B). In addition,  
the variants were tested for inhibition of vitronectin  
25 binding to the receptor. Similar to fibrinogen, the  
variants were more effective at inhibiting the  
40 interaction than the parent molecule. Thus, consistent  
with the cross-reactivity studies with related integrin  
receptors, mutations which increased affinity did not,  
45 appear to substantially alter the manner in which the  
antibody interacted with the receptor.



5 The ability of the variants to inhibit the  
adhesion of M21 human melanoma cells expressing the  $\alpha_v\beta_3$   
receptor to fibrinogen was examined. Inhibition of the  
10 adhesion of  $4 \times 10^4$  M21 cells to fibrinogen by the LM609  
5 grafted antibody variants was performed as described  
previously (Leavesley et al., J. Cell Biol. 117:1101-1107  
(1992)). Similar to the ligand competition studies with  
15 purified fibrinogen and  $\alpha_v\beta_3$  receptor, higher affinity  
variants were generally more effective at preventing cell  
20 adhesion than was LM609 grafted antibody (Figure 11).  
Figure 11 shows inhibition of M21 human melanoma cell  
adhesion to fibrinogen. Cells and various concentrations  
of LM609 grafted antibody Fab (closed triangles), S102  
(open circles), G102 (closed circles), or C37 (open  
25 triangles) were added to 96 well cell culture plates  
which had been coated with 10  $\mu\text{g/ml}$  fibrinogen. After  
incubating for 35 min at 37°C, unbound cells were removed  
by washing and adherent cells were quantitated by crystal  
30 violet staining.

20 Although intact LM609 grafted antibody Ig  
inhibits cell adhesion, the phage expressed Fab did not  
35 affect cell adhesion at concentrations as high as 1 mg/ml  
(Figure 11, closed triangles). Clone C37, isolated from  
the combinatorial library and displaying ~90-fold greater  
25 affinity than LM609 grafted antibody Fab, inhibited cell  
adhesion completely (Figure 11, open triangles). Variant  
40 G102 had a moderately higher affinity (2.2-fold enhanced)  
and also inhibited cell adhesion, though less effectively  
than C37 (Figure 11, closed circles). Surprisingly,  
45 30 clone S102 (Figure 11, open circles), which had a  
4.6-fold higher affinity than LM609 grafted antibody, was  
ineffective at inhibiting cell adhesion, suggesting that  
clones G102 and S102 interact with the  $\alpha_v\beta_3$  receptor  
50 differently.

5                   These results show that combining single amino  
acid mutations that result in LM609 grafted antibodies  
exhibiting higher binding affinity to  $\alpha_v\beta_3$ , allows the  
10                   identification of high affinity LM609 grafted antibody  
5                   mutants having greater than 90-fold higher binding  
affinity than the parent LM609 grafted antibody.

15                   **EXAMPLE VIII**

Generation of High Affinity Enhanced LM609 Grafted  
                  Antibodies

20                   10                   This example describes the generation of high  
affinity enhanced LM609 grafted antibodies having  
increased stability.

25                   The high affinity clone 6H6 (see Table 10 in  
Example VII) was further characterized and was found to  
15                   exhibit some proteolysis. Therefore, to identify  
30                   variants having increased stability, 32 codon variants  
were introduced simultaneously at each of positions Asn<sup>96</sup>  
and His<sup>97</sup> (numbering according to Kabat et al., *supra*) in  
the heavy chain CDR3 of 6H6 (SEQ ID NO:94); and the  
35                   20                   variants were screened for  $\alpha_v\beta_3$  binding activity. Those  
variants that retained binding activity were sequenced  
and then screened for susceptibility to proteolysis. The  
40                   variant 6H6LH was identified as exhibiting  $\alpha_v\beta_3$  binding  
25                   activity and resistance to proteolysis (see Tables 11 and  
15). The 6H6LH variant, which had high affinity  $\alpha_v\beta_3$   
binding activity, had Leu substituted for Asn<sup>96</sup> but  
retained His<sup>97</sup>.

45                   Although the 6H6LH variant exhibited high  
affinity binding to  $\alpha_v\beta_3$ , the affinity was somewhat lower  
50                   30                   than the 6H6 variant (see Table 15). Therefore, CDR

5 mutations that had been found to give high affinity binding were combined with the 6H6LH variant.

Specifically, His<sup>32</sup> in the light chain CDR1 of 6H6 was mutated to Phe, as in clone F32 (see Table 9), to

10 generate clone 2236/6H6LH (see Table 13). In an additional variant, Leu<sup>61</sup> in the heavy chain CDR2 of 6H6 was also mutated to Pro, as in clone P60 (see Table 9),  
15 to generate clone 2236-38/6H6LH (see Table 11).

Shown in Tables 11 and 12 are amino acid and  
10 nucleotide sequences, respectively, of heavy chain CDRs of the enhanced high affinity variants of 6H6. Shown in  
20 Tables 13 and 14 are amino acid and nucleotide sequences, respectively, of light chain CDRs of the enhanced high affinity variants of 6H6. The CDRs shown are according  
25 to Chothia, *supra*. The amino acids and codons that differ from the 6H6 variant are shown in bold.

5

**Table 11: Amino Acid Sequences of Heavy Chain CDRs of  
High Affinity Enhanced 6H6 Variant Antibodies**

10

## Heavy Chain CDR1

## Clone

15

5	6H6LH	G F T F S S Y D M S	SEQ ID NO:34
	2236/6H6LH	G F T F S S Y D M S	SEQ ID NO:34
	2236-38/6H6LH	G F T F S S Y D M S	SEQ ID NO:34

## Heavy Chain CDR2

## Clone

20

10	6H6LH	K V S S G G G S T Y	SEQ ID NO:102
		Y L D T V Q G	
	2236/6H6LH	K V S S G G G S T Y	SEQ ID NO:102
		Y L D T V Q G	
25	2236-38/6H6LH	K V S S G G G S T Y	SEQ ID NO:104
15		Y P D T V Q G	

25

30

## Heavy Chain CDR3

	6H6LH	H L H G S F A S	SEQ ID NO:106
	2236/6H6LH	H L H G S F A S	SEQ ID NO:106
	2236-38/6H6LH	H L H G S F A S	SEQ ID NO:106

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**Table 12: Nucleotide Sequences of Heavy Chain CDRs of  
High Affinity Enhanced 6H6 Variant Antibodies**

Heavy Chain CDR1

6H6LH	GGA TTC ACC TTC AGT AGC TAT GAC	
	ATG TCT	SEQ ID NO:33
2236/6H6LH	GGA TTC ACC TTC AGT AGC TAT GAC	
	ATG TCT	SEQ ID NO:33
2236-38/6H6	GGA TTC ACC TTC AGT AGC TAT GAC	
	ATG TCT	SEQ ID NO:33

Heavy Chain CDR2

6H6LH	AAA GTT AGT AGT GGT GGT GGT AGC	
	ACC TAC TAT TTA GAC ACT GTG CAG	
	GGC	SEQ ID NO:101
2236/6H6LH	AAA GTT AGT AGT GGT GGT GGT AGC	
	ACC TAC TAT TTA GAC ACT GTG CAG	
	GGC	SEQ ID NO:101
2236-38/6H6LH	AAA GTT AGT AGT GGT GGT GGT AGC	
	ACC TAC TAT <b>CCA</b> GAC ACT GTG CAG	
	GGC	SEQ ID NO:103

Heavy Chain CDR3

6H6LH	CAT <b>CTT</b> CAT GGC AGT TTT GCT TCT	
		SEQ ID NO:105
2236/6H6LH	CAT <b>CTT</b> CAT GGC AGT TTT GCT TCT	
		SEQ ID NO:105
2236-38/6H6LH	CAT <b>CTT</b> CAT GGC AGT TTT GCT TCT	
		SEQ ID NO:105

5

**Table 13: Amino Acid Sequences of Light Chain CDRs of  
High Affinity Enhanced 6H6 Variant Antibodies**

10

## Light Chain CDR1

6H6LH Q A S Q S I S N H L H SEQ ID NO:108

5 2236/6H6LH Q A S Q S I S N F L H SEQ ID NO:110

2236-38/6H6LH Q A S Q S I S N F L H SEQ ID NO:110

15

## Light Chain CDR2

6H6LH Y R S Q S I S SEQ ID NO:112

20

2236/6H6LH Y R S Q S I S SEQ ID NO:112

10 2236-38/6H6LH Y R S Q S I S SEQ ID NO:112

## Light Chain CDR3

25

6H6LH Q Q S G S W P L T SEQ ID NO:90

2236/6H6LH Q Q S G S W P L T SEQ ID NO:90

2236-38/6H6LH Q Q S G S W P L T SEQ ID NO:90

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**Table 14: Nucleotide Sequences of Light Chain CDRs of High Affinity Enhanced 6H6 Variant Antibodies**

Light Chain CDR1	
6H6LH	CAG GCC AGC CAA AGT ATT AGC AAC
5	CAC CTA CAC SEQ ID NO:107
2236/6H6LH	CAG GCC AGC CAA AGT ATT AGC AAC
15	TTC CTA CAC SEQ ID NO:109
2236-38/6H6LH	CAG GCC AGC CAA AGT ATT AGC AAC
	TTC CTA CAC SEQ ID NO:109
Light Chain CDR2	
6H6LH	TAT CGT TCC CAG TCC ATC TCT
	SEQ ID NO:111
2236/6H6LH	TAT CGT TCC CAG TCC ATC TCT
	SEQ ID NO:111
15 2236-38/6H6LH	TAT CGT TCC CAG TCC ATC TCT
	SEQ ID NO:111
Light Chain CDR3	
6H6LH	CAA CAG AGT GGC AGC TGG CCT CTG
	ACG SEQ ID NO:89
20 2236/6H6LH	CAA CAG AGT GGC AGC TGG CCT CTG
	ACG SEQ ID NO:89
2236-38/6H6LH	CAA CAG AGT GGC AGC TGG CCT CTG
	ACG SEQ ID NO:89

The kinetics of  $\alpha_v\beta_3$  binding was determined for each clone using surface plasmon resonance (BIAcore) essentially as described in Example VI. The binding kinetics and affinities of the clones are shown in Table 15.

**Table 15. Binding Kinetics of Enhanced LM609 Grafted Antibody Clones**

Clone ID#	$k_{on}$	$k_{off}$	$K_d$
6H6	2.59E+05	1.60E-04	0.62 nM
2236/6H6LH	2.25E+05	1.48E-04	0.66 nM
6H6LH	3.01E+05	5.48E-04	1.82 nM
2236-38/6H6LH	2.76E+05	2.49E-04	0.90 nM

As shown in Table 15, clones 6H6LH, 2236/6H6LH, and 2236-38/6H6LH had affinities similar to 6H6. The affinity for 6H6 in these experiments was 0.62 nM, compared to 0.4 nM observed in the previous experiment (see Table 10 in Example VII). This difference is due to the observed  $k_{on}$ , which is within the variability of the experiments. The on rates were very similar between 6H6, 6H6LH, 2236/6H6LH, and 2236-38/6H6LH. The small difference in the observed  $K_d$  appeared to be due primarily to differences in  $k_{off}$ .

These results describe additional enhanced LM609 grafted antibodies exhibiting high affinity for  $\alpha_v\beta_3$  and resistance to proteolysis.

Throughout this application various publications have been referenced. The disclosures of these publications in their entireties are hereby incorporated by reference in this application in order to more fully describe the state of the art to which this invention pertains.



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Although the invention has been described with reference to the disclosed embodiments, those skilled in the art will readily appreciate that the specific experiments detailed are only illustrative of the invention. It should be understood that various modifications can be made without departing from the spirit of the invention. Accordingly, the invention is limited only by the following claims.

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## Claims

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What is claimed is:

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1. An enhanced LM609 grafted antibody exhibiting selective binding affinity to  $\alpha_v\beta_3$ , or a functional fragment thereof, comprising a CDR selected from the group consisting of a  $V_H$  CDR2 referenced as SEQ ID NO:104; a  $V_H$  CDR3 referenced as SEQ ID NO:106; and a  $V_L$  CDR1 referenced as SEQ ID NO:110.

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2. The enhanced LM609 grafted antibody of claim 1, wherein said functional fragment is selected from the group consisting of Fv, Fab,  $F(ab)_2$  and scFV.

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3. An enhanced LM609 grafted antibody substantially the same as the enhanced LM609 grafted antibody of claim 1.

30

4. An enhanced LM609 grafted antibody exhibiting selective binding affinity to  $\alpha_v\beta_3$ , or a functional fragment thereof, comprising the  $V_H$  CDR1 referenced as SEQ ID NO:34; the  $V_H$  CDR2 referenced as SEQ ID NO:102; the  $V_H$  CDR3 referenced as SEQ ID NO:106; the  $V_L$  CDR1 referenced as SEQ ID NO:108; the  $V_L$  CDR2 referenced as SEQ ID NO:112; and the  $V_L$  CDR3 referenced as SEQ ID NO:90.

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5. The enhanced LM609 grafted antibody of claim 4, wherein said functional fragment is selected from the group consisting of Fv, Fab,  $F(ab)_2$  and scFV.

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6. An enhanced LM609 grafted antibody substantially the same as the enhanced LM609 grafted antibody of claim 4.

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7. An enhanced LM609 grafted antibody exhibiting selective binding affinity to  $\alpha_v\beta_3$ , or a functional fragment thereof, comprising the  $V_H$  CDR1 referenced as SEQ ID NO:34; the  $V_H$  CDR2 referenced as SEQ ID NO:102; the  $V_H$  CDR3 referenced as SEQ ID NO:106; the  $V_L$  CDR1 referenced as SEQ ID NO:110; the  $V_L$  CDR2 referenced as SEQ ID NO:112; and the  $V_L$  CDR3 referenced as SEQ ID NO:90.

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8. The enhanced LM609 grafted antibody of claim 7, wherein said functional fragment is selected from the group consisting of Fv, Fab,  $F(ab)_2$ , and scFV.

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9. An enhanced LM609 grafted antibody substantially the same as the enhanced LM609 grafted antibody of claim 7.

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10. An enhanced LM609 grafted antibody exhibiting selective binding affinity to  $\alpha_v\beta_3$ , or a functional fragment thereof, comprising the  $V_H$  CDR1 referenced as SEQ ID NO:34; the  $V_H$  CDR2 referenced as SEQ ID NO:104; the  $V_H$  CDR3 referenced as SEQ ID NO:106; the  $V_L$  CDR1 referenced as SEQ ID NO:110; the  $V_L$  CDR2 referenced as SEQ ID NO:112; and the  $V_L$  CDR3 referenced as SEQ ID NO:90.

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11. The enhanced LM609 grafted antibody of claim 10, wherein said functional fragment is selected from the group consisting of Fv, Fab,  $F(ab)_2$ , and scFV.

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12. An enhanced LM609 grafted antibody substantially the same as the enhanced LM609 grafted antibody of claim 10.

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13. A nucleic acid molecule encoding the enhanced LM609 grafted antibody of claim 1.

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14. The nucleic acid molecule of claim 13, wherein said nucleic acid molecule comprises a nucleotide sequence selected from the group consisting of SEQ ID NO:103, SEQ ID NO:105, and SEQ ID NO:109.

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15. A nucleic acid molecule encoding the enhanced LM609 grafted antibody of claim 4.

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16. The nucleic acid molecule of claim 15, wherein said nucleic acid molecule comprises the nucleotide sequence referenced as SEQ ID NO:33 encoding a  $V_H$  CDR1; the nucleotide sequence referenced as SEQ ID NO:101 encoding a  $V_H$  CDR2; the nucleotide sequence referenced as SEQ ID NO:105 encoding a  $V_H$  CDR3; the nucleotide sequence referenced as SEQ ID NO:107 encoding a  $V_L$  CDR1; the nucleotide sequence referenced as SEQ ID NO:111 encoding a  $V_L$  CDR2; and the nucleotide sequence referenced as SEQ ID NO:89 encoding a  $V_L$  CDR3.

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17. A nucleic acid molecule encoding the enhanced LM609 grafted antibody of claim 7.

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18. The nucleic acid molecule of claim 17, wherein said nucleic acid molecule comprises the nucleotide sequence referenced as SEQ ID NO:33 encoding a  $V_H$  CDR1; the nucleotide sequence referenced as SEQ ID NO:101 encoding a  $V_H$  CDR2; the nucleotide sequence referenced as SEQ ID NO:105 encoding a  $V_H$  CDR3; the nucleotide sequence referenced as SEQ ID NO:109 encoding a  $V_L$  CDR1; the nucleotide sequence referenced as SEQ ID NO:111 encoding a  $V_L$  CDR2; and the nucleotide sequence referenced as SEQ ID NO:89 encoding a  $V_L$  CDR3.

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19. A nucleic acid molecule encoding the enhanced LM609 grafted antibody of claim 10.

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20. The nucleic acid molecule of claim 19, wherein said nucleic acid molecule comprises the nucleotide sequence referenced as SEQ ID NO:33 encoding a  $V_H$  CDR1; the nucleotide sequence referenced as SEQ ID NO:103 encoding a  $V_H$  CDR2; the nucleotide sequence referenced as SEQ ID NO:105 encoding a  $V_H$  CDR3; the nucleotide sequence referenced as SEQ ID NO:109 encoding a  $V_L$  CDR1; the nucleotide sequence referenced as SEQ ID NO:111 encoding a  $V_L$  CDR2; and the nucleotide sequence referenced as SEQ ID NO:89 encoding a  $V_L$  CDR3.

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21. A method of inhibiting a function of  $\alpha_v\beta_3$ , comprising contacting  $\alpha_v\beta_3$  with the enhanced LM609 grafted antibody of claim 1.

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22. A method of inhibiting a function of  $\alpha_v\beta_3$ , comprising contacting  $\alpha_v\beta_3$  with the enhanced LM609 grafted antibody of claim 4.

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23. A method of inhibiting a function of  $\alpha_v\beta_3$ , comprising contacting  $\alpha_v\beta_3$  with the enhanced LM609 grafted antibody of claim 7.

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24. A method of inhibiting a function of  $\alpha_v\beta_3$ , comprising contacting  $\alpha_v\beta_3$  with the enhanced LM609 grafted antibody of claim 10.

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25. An antibody, or a functional fragment thereof, comprising a CDR selected from the group consisting of a  $V_H$  CDR2 referenced as SEQ ID NO:104; a  $V_H$  CDR3 referenced as SEQ ID NO:106; and a  $V_L$  CDR1 referenced as SEQ ID NO:110, and exhibiting enhanced binding affinity to  $\alpha_v\beta_3$ , compared to LM609.

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26. The antibody of claim 25, wherein said functional fragment is selected from the group consisting of Fv, Fab, F(ab)<sub>2</sub> and scFV.

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27. An antibody, or functional fragment thereof, comprising the  $V_H$  CDR1 referenced as SEQ ID NO:34; a  $V_H$  CDR2 referenced as SEQ ID NO:102; a  $V_H$  CDR3 referenced as SEQ ID NO:106; a  $V_L$  CDR1 referenced as SEQ ID NO:108; a  $V_L$  CDR2 referenced as SEQ ID NO:112; and a  $V_L$  CDR3 referenced as SEQ ID NO:90, and exhibiting enhanced binding activity to  $\alpha_v\beta_3$ , compared to LM609.

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28. The antibody of claim 27, wherein said functional fragment is selected from the group consisting of Fv, Fab, F(ab)<sub>2</sub> and scFV.

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29. An antibody, or a functional fragment thereof, comprising a  $V_H$  CDR1 referenced as SEQ ID NO:34; a  $V_H$  CDR2 referenced as SEQ ID NO:102; a  $V_H$  CDR3 referenced as SEQ ID NO:106; a  $V_L$  CDR1 referenced as SEQ ID NO:110; a  $V_L$  CDR2 referenced as SEQ ID NO:112; and a  $V_L$  CDR3 referenced as SEQ ID NO:90, and exhibiting enhanced binding activity to  $\alpha_v\beta_3$ , compared to LM609.

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5 30. The enhanced LM609 grafted antibody of  
claim 29, wherein said functional fragment is selected  
10 from the group consisting of Fv, Fab, F(ab)<sub>2</sub> and scFV.

31. An antibody, or a functional fragment  
5 thereof, comprising a V<sub>H</sub> CDR1 referenced as SEQ ID NO:34;  
a V<sub>H</sub> CDR2 referenced as SEQ ID NO:104; a V<sub>H</sub> CDR3  
15 referenced as SEQ ID NO:106; a V<sub>L</sub> CDR1 referenced as SEQ  
ID NO:110; a V<sub>L</sub> CDR2 referenced as SEQ ID NO:112; and a V<sub>L</sub>  
CDR3 referenced as SEQ ID NO:90, and exhibiting enhanced  
10 binding activity to  $\alpha_v\beta_3$ , compared to LM609.

32. The enhanced LM609 grafted antibody of  
claim 30, wherein said functional fragment is selected  
25 from the group consisting of Fv, Fab, F(ab)<sub>2</sub> and scFV.

33. A nucleic acid molecule having a  
15 nucleotide sequence selected from the group of nucleotide  
sequences consisting of SEQ ID NO:33, SEQ ID NO:89, SEQ  
30 ID NO:101; SEQ ID NO:103, SEQ ID NO:105, SEQ ID NO:107,  
SEQ ID NO:109, and SEQ ID NO:111.



CAG GTG CAG CTG GTG GAG TCT GGG GGA GGC GTT GTG CAG CCT GGA AGG 48  
 Gln Val Gln Leu Val 5 Gly Gly Val Val Val Gln Pro Gly Arg 15  
 TCC CTG AGA CTC TCC TGT GCA GCC TCT GGA TTC ACC TTC AGT AGC TAT 96  
 Ser Leu Arg Leu Ser Cys Ala Ala Ser Gly Phe Thr Phe Ser Ser Tyr 30  
 GAC ATG TCT TGG GTT CGC CAG GCT CCG GGC AAG GGT CTG GAG TGG GTC 144  
 Asp Met Ser Ser Trp Val Arg Gln Ala Pro Gly Lys Gly Leu Glu Trp Val 45  
 GCA AAA GTT AGT AGT GGT GGT AGC ACC TAC TAT TTA GAC ACT GTG 192  
 Ala Lys Val Ser Ser Gly Gly Ser Thr Tyr Tyr Leu Asp Thr Val 60  
 CAG GGC CGA TTC ACC ATC TCC AGA GAC AAT AGT AAG AAC ACC ACC CTA TAC 240  
 Gln Gly Arg Phe Thr Ile Ser Arg Asp Asn Ser Lys Asn Thr Leu Tyr 75  
 CTG CAA ATG AAC TCT CTG AGA GCC GAG GAC ACA GGC GTG TAT TAC TGT 288  
 Leu Gln Met Asn Ser Leu Arg Ala Glu Asp Thr Ala Val Tyr Tyr Cys 95  
 GCA AGA CAT AAC TAC GGC AGT TTT GCT TAC TGG GGC CAA GGG ACT ACA 336  
 Ala Arg His Asn Tyr Gly Ser Phe Ala Tyr Trp Gly Gln Gly Thr Thr 110  
 GTG ACT GTT TCT AGT 351  
 Val Thr Val Ser Ser 115

FIG. 1A

GAG ATT GTG CTA ACT CAG TCT CCA GCC ACC CTG TCT CTC AGC CCA GGA 48  
 Glu Ile Val Leu Thr Gln Ser Pro Ala Thr Leu Ser Leu Ser Pro Gly 15  
 GAA AGG GCG ACT CTT TCC TGC CAG GCC AGC CAA AGT ATT AGC AAC CAC 96  
 Glu Arg Ala Thr Leu Ser Cys Gln Ala Ser Gln Ser Ile Ser Asn His 30  
 CTA CAC TGG TAT CAA CAA AGG CCT GGT CAA GCC CCA AGG CTT CTC ATC 144  
 Leu His Trp Tyr Gln Gln Arg Pro Gly Gln Ala Pro Arg Leu Leu Ile 45  
 AAG TAT CGT TCC CAG TCC ATC TCT GGG ATC CCC GCC AGG TTC AGT GGC 192  
 Lys Tyr Arg Ser Gln Ser Ile Ser Gly Ile Pro Ala Arg Phe Ser Gly 60  
 AGT GGA TCA GGG ACA GAT TTC ACC CTC ACT ATC TCC AGT CTG GAG CCT 240  
 Ser Gly Ser Gly Thr Asp Phe Thr Leu Thr Ile Ser Ser Leu Glu Pro 75  
 GAA GAT TTT GCA GTC TAT TAC TGT CAA CAG AGT GGC AGC TGG CCT CAC 288  
 Glu Asp Phe Ala Val Tyr Tyr Cys Gln Gln Ser Gly Ser Trp Pro His 85  
 ACG TTC GGA GGG GGG ACC AAG GTG GAA ATT AAG 321  
 Thr Phe Gly Gly Gly Thr Lys Val Glu Ile Lys 105

FIG. IB

48  
 GAA GTG CAG CTG GTG GAG TCT GGG GGA GGC TTA GTG AAG CCT GGA AGG  
 Glu Val Gln Gln Leu Val<sup>5</sup> Gly Gly<sup>10</sup> Leu Val Lys Pro Gly Arg<sup>15</sup>  
 96  
 TCC CTG AGA CTC TCC TGT GCA GCC TCT GGA TTC GCT TTC AGT AGC TAT  
 Ser Leu Arg<sup>20</sup> Leu Ser Cys Ala Ala Ser<sup>25</sup> Gly Phe Ala Phe Ser Ser Tyr<sup>30</sup>  
 144  
 GAC ATG TCT TGG GTT CGC CAG ATT CCG GAG AAG AGG CTG GAG TGG GTC  
 Asp Met Ser<sup>35</sup> Trp Val Arg Gln Ile Pro Glu Lys Arg<sup>40</sup> Leu Glu Trp Val<sup>45</sup>  
 192  
 GCA AAA GTT AGT AGT GGT GGT GGT AGC ACC TAC TAT TTA GAC ACT GTG  
 Ala Lys Val Ser Ser Gly<sup>50</sup> Gly Ser Thr Tyr<sup>55</sup> Tyr Leu Asp Thr Val<sup>60</sup>  
 240  
 CAG GGC CGA TTC ACC ATC TCC AGA GAC AAT GCC AAG AAC ACC CTA TAC  
 Gln Gly Arg Phe Thr<sup>65</sup> Ile Ser Arg Asp Asn Ala<sup>70</sup> Lys Asn Thr Leu Tyr<sup>75</sup>  
 288  
 CTG CAA ATG AGC AGT CTG AAC TCT GAG GAC ACA GCC ATG TAT TAC TGT  
 Leu Gln Met Ser Ser<sup>80</sup> Leu Asn Ser Glu Asp<sup>85</sup> Thr Ala Met Tyr Tyr Cys<sup>90</sup>  
 336  
 GCA AGA CAT AAC TAC GGC AGT TTT GCT TAC TGG GGC CAA GGG ACT CTG  
 Ala Arg His<sup>100</sup> Asn Tyr Gly Ser Phe Ala Tyr Trp Gly Gln<sup>105</sup> Gly Thr Leu<sup>110</sup>  
 351  
 GTC ACT GTC TCT GCA  
 Val Thr<sup>115</sup>

FIG. 2A

GAT ATT GTG CTA ACT CAG TCT CCA GCC ACC CTG TCT GTG ACA CCA GGA 48  
 Asp Ile Val Leu Thr Gln Ser Pro Ala Thr Pro Gly  
 1 5 10 15  
 GAT AGC GTC AGT CTT TCC TGC CAG GCC AGC CAA AGT ATT AGC AAC CAC 96  
 Asp Ser Val Ser Leu Ser Cys Gln Ala Ser Gln Ser Ile Ser Asn His  
 20 25 30  
 CTA CAC TGG TAT CAA CAA AAA TCA CAT GAG TCT CCA AGG CTT CTC ATC 144  
 Leu His Trp Tyr Gln Gln Lys Ser His Glu Ser Pro Arg Leu Leu Ile  
 35 40 45  
 AAG TAT CGT TCC CAG TCC ATC TCT GGG ATC CCC TCC AGG TTC AGT GGC 192  
 Lys Tyr Arg Ser Gln Ser Ile Ser Gly Ile Pro Ser Arg Phe Ser Gly  
 50 55 60  
 AGT GGA TCA GGG ACA GAT TTC GCT CTC AGT ATC AAC AGT GTG GAG ACT 240  
 Ser Gly Ser Gly Thr Asp Phe Ala Leu Ser Ile Asn Ser Val Glu Thr  
 65 70 75 80  
 GAA GAT TTT GGA ATG TAT TTC TGT CAA CAG AGT GGC AGC TGG CCT CAC 288  
 Glu Asp Phe Gly Met Tyr Phe Cys Gln Gln Ser Gly Ser Trp Pro His  
 85 90 95  
 ACG TTC GGA GGG GGG ACC AAG CTG GAA ATT AAG 321  
 Thr Phe Gly Gly Gly Thr Lys Lys Leu Glu Ile Lys  
 100 105

FIG. 2B

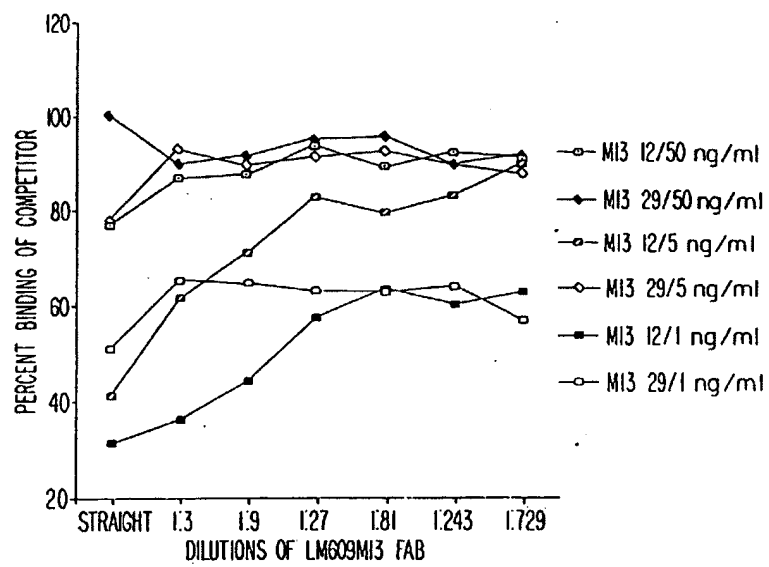


FIG. 3

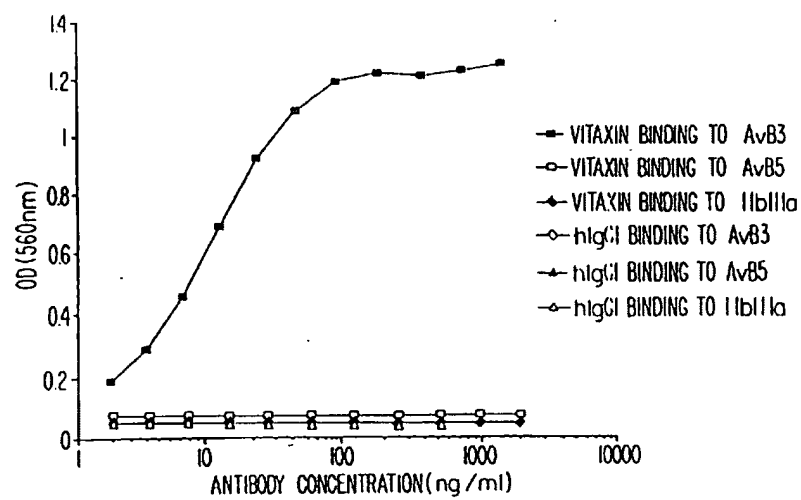


FIG. 4A

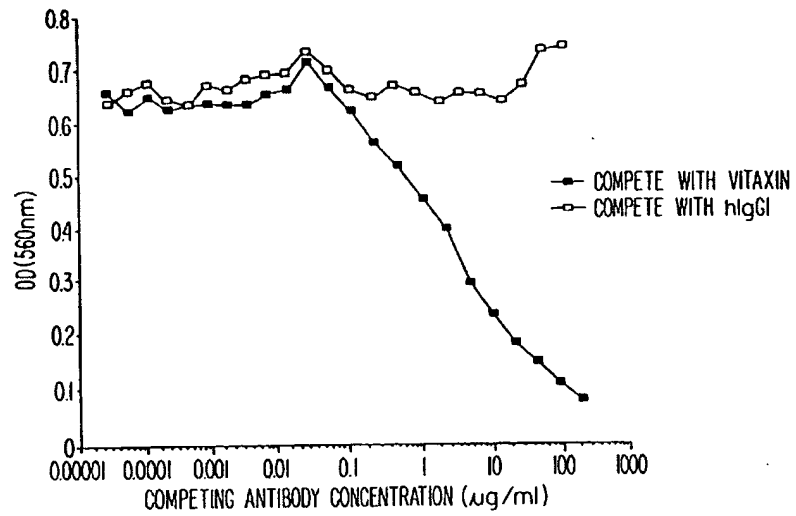


FIG. 4B

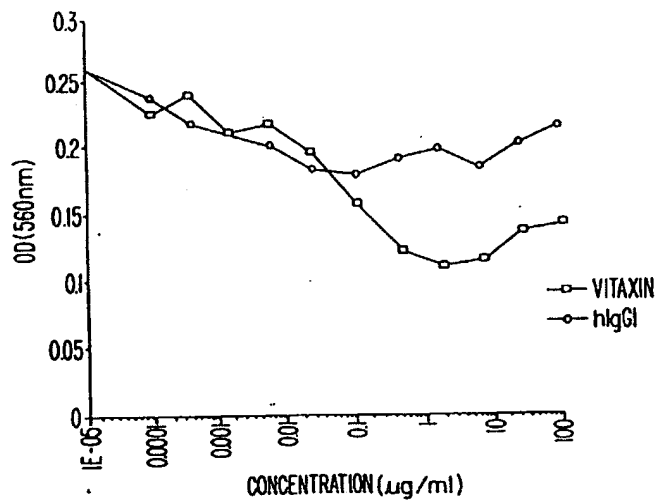


FIG. 4C

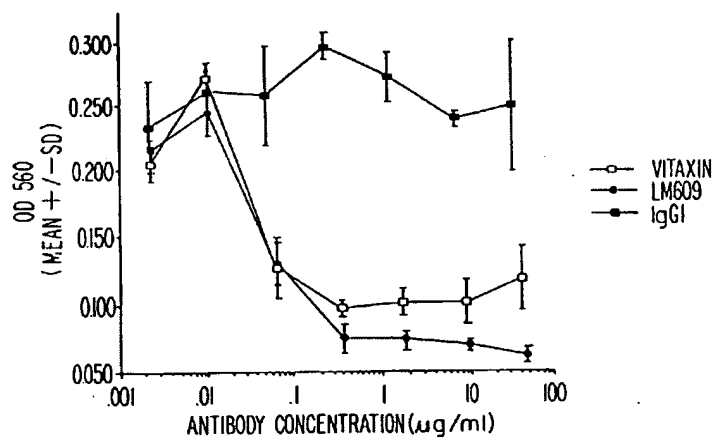


FIG. 5A

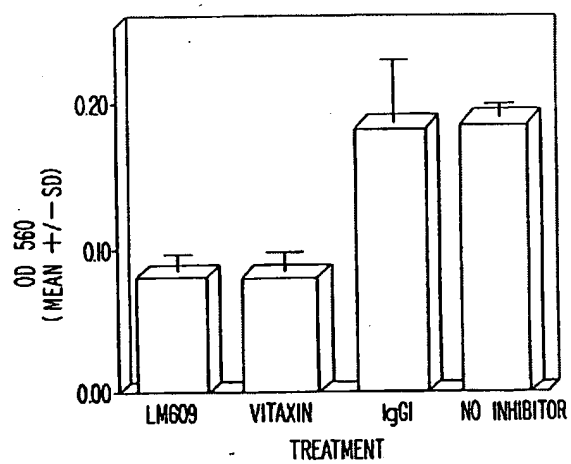


FIG. 5B



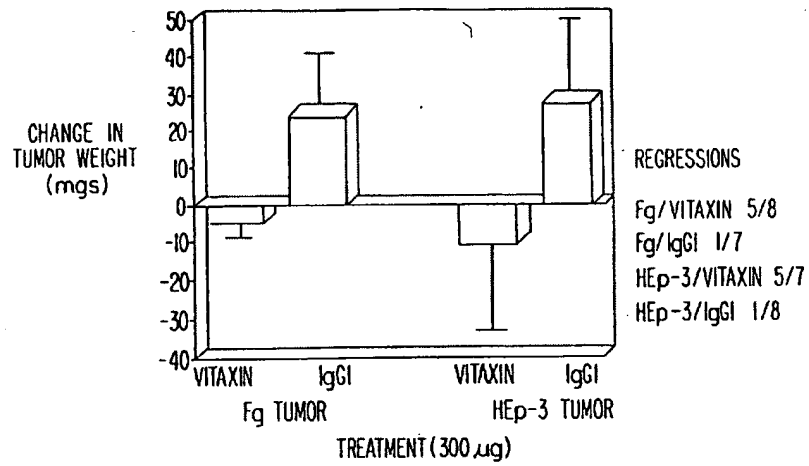


FIG. 6A

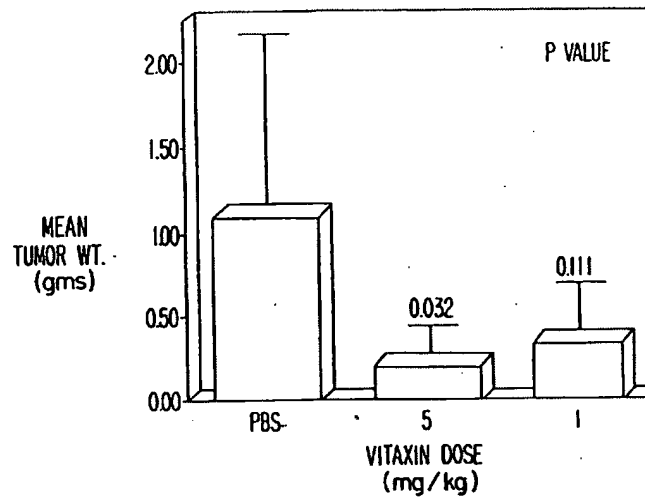


FIG. 6B

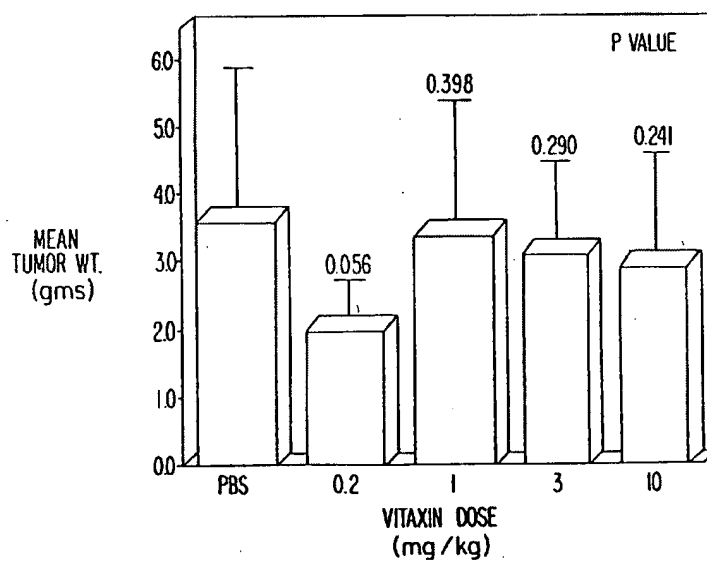


FIG. 6C

48 GAG ATT GTG CTA ACT CAG TCT CCA GCC ACC CTG TCT CTC AGC CCA GGA  
 Glu Ile Val Leu Thr 5  
 96 GAA AGG GCG ACT CTT TCC TGC CAG GCC AGC CAA AGT ATT AGC AAC CAC  
 Glu Arg Ala Thr Leu Ser Cys Gln Ala 25  
 144 CTA CAC TGG TAT CAA CAA AGG CCT GGT CAA GCC CCA AGG CTT CTC ATC  
 Leu His Trp Tyr Gln Arg Pro Gly Gln Ala Pro 45  
 192 CGT/ATG TAT CGT TCC CAG TCC ATC TCT GGG ATC CCC GCC AGG TTC AGT GGC  
 Arg/Met Tyr Arg Ser Gln Ser 55  
 240 AGT GGA TCA GGG ACA GAT TTC ACC CTC ACT ATC TCC AGT CTG GAG CCT  
 Ser Gly Ser Gly Thr Asp Phe Thr Leu Thr 75  
 288 GAA GAT TTT GCA GTC TAT TAC TGT CAA CAG AGT GGC AGC TGG CCT CAC  
 Glu Asp Phe Ala Val Tyr Tyr Cys Gln 90  
 321 ACG TTC GGA GGG GGG ACC AAG GTG GAA ATT AAG  
 Thr Phe Gly Gly Thr Lys Val Glu Ile Lys 105

FIG. 7

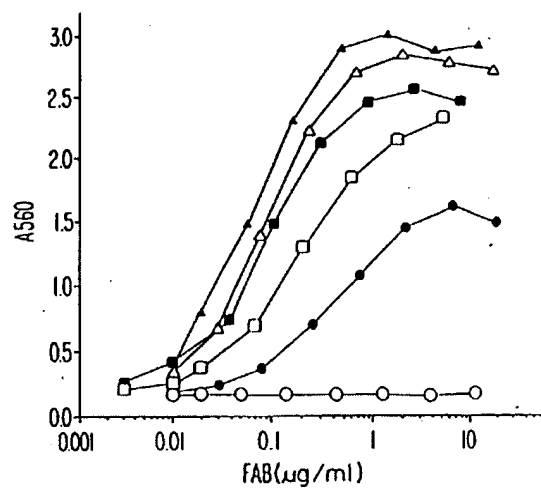


FIG. 8

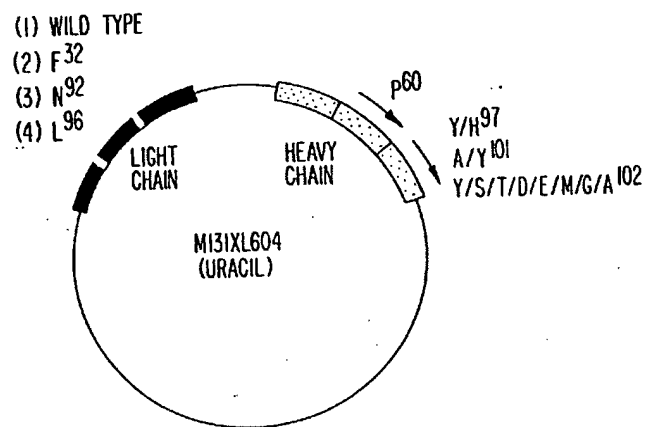


FIG. 9

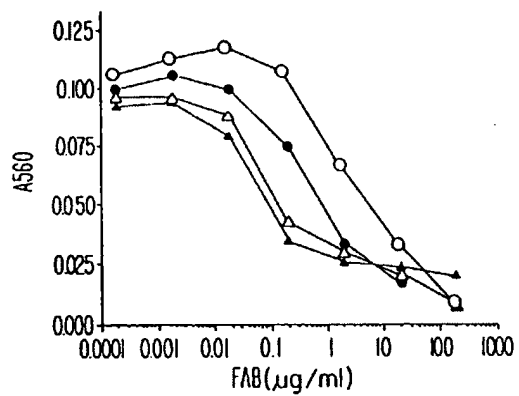


FIG. IOA

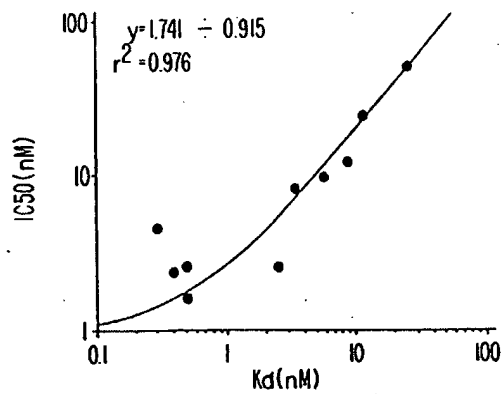


FIG. IOB

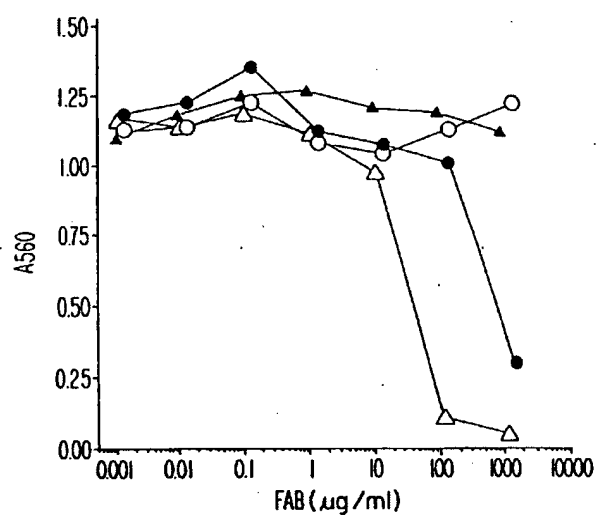


FIG. 11

## INTERNATIONAL SEARCH REPORT

International Application No.

PCT/US 00/17454

A. CLASSIFICATION OF SUBJECT MATTER  
IPC 7 C07K16/28 A61K39/395 C07K16/46 C12N15/13

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 7 C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

EPO-Internal, BIOSIS, WPI Data, PAJ, MEDLINE

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	WO 98 33919 A (IXSYS INC) 6 August 1998 (1998-08-06) abstract page 1, line 8-12 page 5, line 8-12 page 8, line 27-30 page 22, line 1 -page 31, line 32 page 35, line 10 -page 39, line 3 page 39, line 17 -page 45, line 24 page 50; tables 4,5 page 51, line 29 -page 54, line 3 page 82, line 4 -page 100, line 24 --- -/--	1-33



Further documents are listed in the continuation of box C.



Patent family members are listed in annex.

## \* Special categories of cited documents:

\*A\* document defining the general state of the art which is not considered to be of particular relevance

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\*L\* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

\*O\* document referring to an oral disclosure, use, exhibition or other means

\*P\* document published prior to the international filing date but later than the priority date claimed

\*T\* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

\*X\* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

\*Y\* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

\*A\* document member of the same patent family

Date of the actual completion of the international search

10 November 2000

Date of mailing of the international search report

20. 11. 00

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Authorized officer

Montrone, M



## INTERNATIONAL SEARCH REPORT

International Application No  
PCT/US 00/17454

## C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	WU HERREN ET AL: "Stepwise in vitro affinity maturation of Vitaxin, an alphavbeta3-specific humanized mAb." PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF THE UNITED STATES, vol. 95, no. 11, 26 May 1998 (1998-05-26), pages 6037-6042, XP000941358 May 26, 1998 ISSN: 0027-8424 abstract page 6037, column 2, paragraph 2 -page 6038, column 1, paragraph 1	1-33
Y	DAVIES JULIAN ET AL: "Affinity improvement of single antibody VH domains: Residues in all three hypervariable regions affect antigen binding." IMMUNOTECHNOLOGY (AMSTERDAM), vol. 2, no. 3, 1996, pages 169-179, XP004070292 ISSN: 1380-2933 abstract	1-33
Y	CHOWDHURY P S ET AL: "Improving antibody affinity by mimicking somatic hypermutation in vitro." NATURE BIOTECHNOLOGY, (1999 JUN) 17 (6) 568-72., XP000918985 abstract	1-33

# INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US 00/17454

## Box I Observations where certain claims were found unsearchable (Continuation of Item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.:  
because they relate to subject matter not required to be searched by this Authority, namely:  
Although claims 21 to 24 are partially directed to a method of treatment of the human/animal body insofar as they refer to an in vivo method, the search has been carried out and based on the alleged effects of the compound.
2. ☐ Claims Nos.:  
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:
3. ☐ Claims Nos.:  
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

## Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

### Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.  
☐ No protest accompanied the payment of additional search fees.

# INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/US 00/17454

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9833919 A	06-08-1998	AU 6139198 A EP 0970217 A	25-08-1998 12-01-2000